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**SECONDARY METABOLITES FROM
XYLARIACEOUS FUNGI**

R. A. A. ALHAIDARI

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SECONDARY METABOLITES FROM *XYLARIACEOUS* FUNGI

The isolation and structure elucidation of secondary metabolites from
Xylariaceae fungi by chemical and spectroscopic methods

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Abstract

Keywords: Endophytes, *Xylaria*, fungi, secondary metabolites, NMR, structure elucidation.

This thesis describes the isolation and structure elucidation of secondary metabolites formed in static culture from a number of endophytic *Xylariaceae* fungi. Four *Xylaria* endophytes isolated from a palm tree in Thailand were surface cultured on an aqueous malt extract-glucose medium. They all produced cytochalasin D, coriloxin, (*S*)-mellein and (3*R*,4*R*)-4-hydroxymellein as the main secondary metabolites suggesting that the four endophytes could be the same species. The endophytic fungus **A116** produced cytochalasin D as the main secondary metabolite. Another non-endophytic fungus **B315**, produced cytochalasin D, (*R*)-mellein, a mixture of two isomers of 4-hydroxymellein and phloroglucinol. **X.62**, an endophytic fungus, produced 19,20-epoxycytochalasin C from the mycelium as the main secondary metabolite. The fungus *Engleromyces sinensis* produced engleromycin acetate as the main secondary metabolite. Fungus *X. polymorpha* produced (3*E*)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid.

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Glossary

°C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
[α]	Specific rotatory power
δ	NMR chemical shift [ppm]
ν	Wave number [cm^{-1}]
br	Broad
c	Concentration [g/100 mL]
cm	10^{-2} metre
COSY	Correlated Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement by Polarisation Transfer
e.g.	Example given
ES	Electrospray
g	Gram
HMBC	Hetero nuclear Multiple Bond Correlation
HMQC	Hetero nuclear Multiple Quantum Coherence
Hz	Hertz
IR	Infrared
J	Spin-spin coupling constant [Hz]
L	Litre
Lit	Literature
m	Multiplet (in connection with NMR data)
MeOH	Methanol
MHz	Megahertz
mp	Melting point

MS	Mass Spectrometry
m/z	Molecular Ion
NMR	Nuclear Magnetic Resonance
PLC	Preparative Layer Chromatography
ppm	parts per million
q	Quartet
s	Singlet
sp.	Species
t	Triplet
TLC	Thin Layer Chromatography
UV	Ultra Violet
XRD	X-ray diffraction
R_f	values refere to migration relative to the solvent front

Anamorph	the asexual reproductive manifestation of a fungus.
Ascospore	a spore born in asci.
Conidia	unicellular asexual reproductive spore produced externally.
Conidiogenesis	the process of conidia formation.
Holoblastic	the process of undergoing complete segregation.
Teleomorph	the sexual manifestation of a fungus.
Genus	a taxonomic category that includes a number of species.
Stroma	mass of fungal tissue within which or from which perithecia are form.
Chemotaxonomy	the classification & identification of fungi based on profiles of secondary metabolites.
Mycelium	collective term for hyphae; the vegetative thallus of a fungus excluding organs of sporulation.

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Chapter 1 Introduction

1.1 The Family *Xylariaceae*

The *Xylariaceae* is a family of fungi belonging to the *Ascomycota* which traditionally has been divided into three main subclasses, *Plectomycetes*, *Discomycetes* and *Pyrenomycetes*. The *Xylariaceae* belongs to the *Pyrenomycetes* subclass. This family comprises of at least 40 genera¹⁻⁴, but nearly 70 genera have now been listed⁵. Although *Xylariaceous* fungi occupy a wide range of habitats, the majority are wood inhabitants⁶. These saprophytic fungi can be found on decayed wood, litter, fruits, seeds, dung and leaves and on insects^{7, 8}.

Many studies have revealed that members of *Xylariaceae* occur as endophytes⁸⁻¹⁰; a fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of diseases¹⁰. Studies have shown that endophytes are a promising source of novel natural metabolites, which exhibit a variety of biological activities¹¹. Such metabolites have potential use as pharmaceuticals.

Over the past 25 years, the *Xylariaceae* have received considerable attention and as a result there is now a clear understanding of its constituent genera. In 1979 using morphological data, Rogers¹² proposed a central core of closely related genera of family *Xylariaceae* comprising: *Xylaria*, *Hypoxylon*, *Rosellinia*, *Poronia*, *Hypocopra*, *Daldinia*, *Camillea*, *Penzigia*, *Nummularia* and *Kretzschmaria*. In 1995 Whalley and Edwards¹³ accepted the concept of core genera as proposed by Rogers. In recent years Whalley⁶ proposed a scheme illustrating the relationships between these genera. In this scheme he

related *Xylaria* to *Hypoxylon* and *Nummularia*. He thought that *Poronia* is more related to *Xylaria*, whereas *Rosellinia* is closely related to *Nemania*.

The taxonomic classification of fungi species has been attempted in different ways. This includes use of:

- mycological data^{14, 15}
- chemical data^{6, 16}
- DNA profiling^{6, 17}

1.2 The Genus *Xylaria*

Xylaria is a complex and the oldest known genus of the family *Xylariaceae*. *Xylaria* species have a wide geographical distribution throughout the tropics, subtropics and temperate regions and more than 500 species are believed to belong to this genus¹⁸. *Xylaria* species are considered as saprophytes or weak parasites¹⁹. Other *Xylariaceae* species are pathogens such as *Entoleuca* (*Hypoxylon mammata*), which is a well known cause of canker disease⁶.

In spite of the fact that *Xylaria* are widely available, their classification is often confused. This is due to their variation in shape, colour, and size and even in form of the stroma depending on their level of maturity, host and environment. Stromatal morphology has been the basis of classification of the *Xylaria* species by early researchers^{20, 21}. Rogers^{22, 23} **Table 1**, divided the *Xylaria* into four sections based on the place of their anamorph in their life cycle and the morphological structure of their conidiogenous and stromata (telemorph). Although Rogers's classification is more reliable, it is incomplete, and many gaps need to be filled because not all the species have been cultured and few *Xylaria* species have anamorphic data. At the present there

is no stable reliable taxonomic system for *Xylaria* sub-genera. The use of chemotaxonomy could help to highlight the relations between the different species²⁴.

Table 1 Sub-division of *Xylaria* genera according to Rogers^{22, 23}

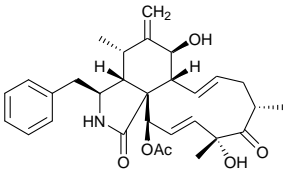
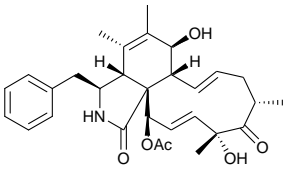
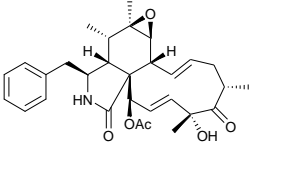
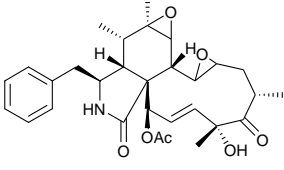
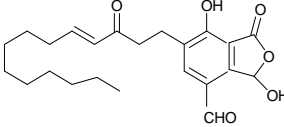
Section	Characteristic features
1	Conidia produced from palisade of conidiogenous cells over active young teleomorphic stromata or at least not limited to specialised appendages, conidiogenesis holoblastic. Conidia produced in more or less sympodial sequence, seeding individually and passively. This section contains 5 groups: <i>Xylaria polymorpha</i> , <i>X. hypoxylon</i> , <i>X. multiplex</i> , <i>X. pyramidata</i> and <i>X. pedunculata</i> .
2	Conidia produced on special and localised peg or hairy-shaped appendages on young teleomorphic stromata, conidia apparently seeding individually and passively. This section is represented by the <i>X. comosa</i> group.
3	Conidia produced on special anamorphic stromata or conidiomata which usually produced earlier in the year than teleomorphic stromata. Teleomorphic stromata never bear conidia, conidiogenesis holoblastic. Conidia produced in more or less sympodial sequence, seeding individually and passively, e.g. <i>X. cubensis</i> .
4	Conidia produced on young teleomorphic stromata. Conidiogenesis apparently holoblastic. Conidia produced in tandem, discharging forcibly. This section is represented by <i>X. furcata</i> .

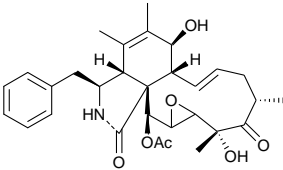
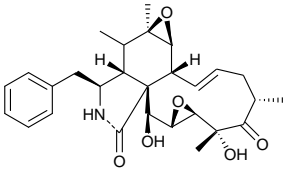
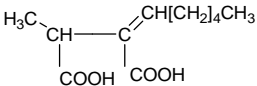
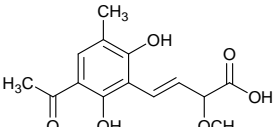
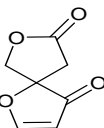
1.3 Secondary metabolites from *Xylariaceous* fungi

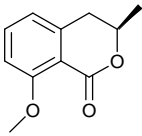
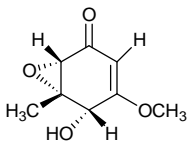
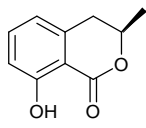
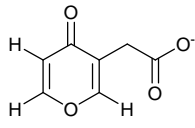
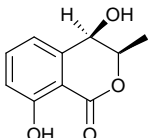
A fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus. The chemical profile can be used as a taxonomic tool in the classification and identification of filamentous fungi and a big advantage of filamentous *Ascomycetes* is that they can be grown under standardised and controlled laboratory conditions. A satisfactory production of secondary metabolites depends on the medium the fungi are cultured on. The more complex a medium is the more chemical diversity the fungi will display¹⁷. For comparative purposes, fungal cultures scheduled for chemotaxonomic analysis must always be grown on the same medium, incubated at the same temperature, and extracted in the same way, to ensure the differences are due to fungal diversity and not growth conditions.

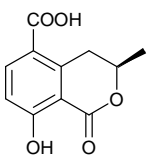
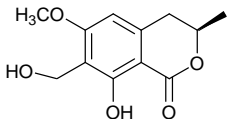
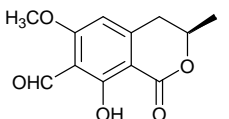
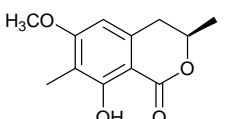
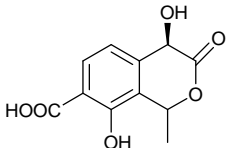
It is not unusual that different fungal species have one or more secondary metabolites in common. Cytochalasin D is one such metabolite. It is produced by several fungal species²⁵⁻²⁷. Secondary metabolites that have been isolated from various *Xylaria* species within the family *Xylariaceae* are presented in **Table 2**.

Table 2 Secondary metabolites from *Xylaria* species

No	Compound structure	<i>Xylaria</i> species
1	 <p>Cytochalasin D</p>	<i>X. cubensis</i> ²⁸ <i>X. hypoxylon</i> ²⁵
2	 <p>Cytochalasin C</p>	<i>X. hypoxylon</i> ²⁵
3	 <p>Cytochalasin Q</p>	<i>X. obovata</i> ²⁹ <i>X. hypoxylon</i> ³⁰
4	 <p>Cytochalasin R</p>	<i>X. hypoxylon</i> ³⁰
5	 <p>Xylaral</p>	<i>X. polymorpha</i> ²⁷

6	 <p>19,20-epoxycytochalasin C</p>	<i>X. obovata</i> ²⁹ <i>X. hypoxylon</i> ³⁰
7	 <p>19,20-epoxycytochalasin Q</p>	<i>X. hypoxylon</i> ³⁰
8	 <p>2-Hexylidene-3-methylsuccinic acid</p>	<i>X. polymorpha</i> ³¹ <i>X. hypoxylon</i> ³¹ <i>X. longipes</i> ³² <i>X. myosurus</i> ³³
9	 <p>Globoscinic acid</p>	<i>X. globosa</i> ³⁴ <i>X. obovata</i> ³⁴
10	 <p>Longianone</p>	<i>X. longiana</i> ³³

11	 <p>S-8-O-methylmellein</p>	<i>X. species</i> ³⁵
12	 <p>Coriloxin</p>	<i>X. species</i> ^{35, 36} <i>X. obovata</i> ³⁴ <i>X. badia</i> ³⁷
13	 <p>Mellein</p>	(S) isomer: <i>X. grammica</i> ^{35, 38-41} (R) isomer: <i>X. longiana</i> ³³ <i>X. badia</i> ³⁷ <i>X. species</i> ³⁵
14	 <p>Xylaric acid</p>	<i>X. species</i> ⁴²
15	 <p>4-Hydroxymellein</p>	<i>X. grammica</i> ³⁵ <i>X. longiana</i> ¹⁸

16	 <p>5-Carboxymellein</p>	<i>X. species</i> ³⁵ <i>X. myosurus</i> ³³
17	 <p>8-Hydroxy-7-hydroxymethyl-6-methoxy-3-methylisocoumarin</p>	<i>X. species</i> ³⁵
18	 <p>7-Formyl-8-hydroxy-6-methoxy-3-methylisocoumarin</p>	<i>X. species</i> ³⁵
19	 <p>3,7-Dimethyl-8-hydroxy-6-methoxyisocoumarin</p>	<i>X. species</i> ³⁵
20	 <p>4,8-Dihydroxy-1-methyl-3-oxo-isochroman-7-carboxylic acid</p>	<i>X. species</i> ³⁵

1.4 Endophytes

Endophytes (Gr. endo, within; phyton, plant) the term was first introduced by de Bary in 1866 and has been deeply embedded in the literature ever since^{43, 44}. Carroll⁴⁵ in 1988 re-defined endophytes as fungi that exist within leaves and stems of healthy plants. In 1991, Petrini⁴⁶, improved Carroll's definition stating that endophytes are organisms, that colonize internal plant tissues at least sometime in their life without causing apparent disease symptoms to the host.

An endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter-cellularly inside the healthy tissues of the host plant without causing any immediate, overt negative effects¹⁰. The most frequently isolated endophytes are fungi⁴⁷. Examination of plant material can lead to the discovery of endophytic fungi and bacteria. The fungi isolated are often host specific⁴⁸, so it is possible, that of the nearly 300,000 plant species that exist on the earth, each individual plant could be host to one or more endophytes^{10, 11}. The number of endophytic species potentially associated with plants can reach several hundred⁴⁹. Furthermore, it has been reported, that endophytes are found in marine algae⁵⁰, mosses and ferns^{51, 52}.

The environmental conditions under which the host is growing influence the endophyte diversity, and the endophyte profile may be more diversified in tropical areas¹⁰. Researchers recently noted that endophytes from tropical regions produced significantly more bioactive secondary metabolites than those from temperate parts of the world^{53, 54}. The question of how microbial endophytes get into their host plants is still not clear. Fungal endophytes can gain access through the roots, but more are believed to enter through wounds in aerial parts of the plant or via stomata and bacterial endophytes are

not thought to invade plant tissue directly; instead, they tend to enter the plant through natural openings or wounds⁵⁵.

1.5 Endophytes and their host relationship

Endophyte-infected plants often grow faster than non-infected ones. This effect is due to the endophyte's production of phytohormones and other plant growth-promotion substances¹⁰. Endophytes may also enhance the hosts uptake of nutritional elements such as nitrogen⁵⁶ and phosphorus⁵⁷⁻⁵⁹. Additionally, some endophytes are able to enhance the host plant tolerance to environmental stresses¹⁰, provide protection against nematodes^{60, 61} and insect herbivores^{62, 63} as well as bacteria and fungal pathogens^{64, 65}.

1.6 Natural products and drug discovery

There is an increase in the number of people in the world having health problems caused by different diseases such as cancer, heart disease, drug-resistant bacteria and parasite infections. An intensive search for new and more effective agents to treat these diseases is under way and fungi, particularly endophytes, are often a novel source of potentially useful medicinal compounds⁶⁶.

Treatments are needed for epidemic human diseases such as AIDS and other severe acute respiratory syndromes. Furthermore, the development of resistance to infectious microorganisms⁶⁷ (e.g. *Staphylococcus*, *Mycobacterium* and *Streptococcus*), life threatening viruses and the recurring problems with diseases in persons with organ transplants. In addition, the world's arsenal is not large for the treatment of parasitic protozoan infections, e.g. malaria, leishmaniasis and filariasis, which probably claim more lives each year than any other group of infectious agents, emphasizes the strong

need for new antibiotics⁴⁷, chemotherapeutic agents and agrochemicals that are highly effective, possess low toxicity with less harmful effects on the environment. Novel natural products and the organisms that make them, offer opportunities for innovation in drug and agrochemical discovery².

It appears that an enormous, untapped source of microbial diversity is represented by the microbial endophytes, which produce various antimicrobial agents. The forests of the world are sources of these microbial endophytes (fungi and bacteria). There are numerous compounds of fungal metabolites, which are derived from various fungi of beneficial use in the modern medicine, agriculture and industry.

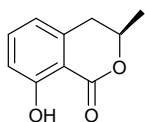
Cyclosporin⁶⁶, is a powerful immunosuppressant used in immunocompromised patients. Other fungi produce alkaloids⁶⁶ that are used in the treatment of migraine. Griseofulvin⁶⁸, is a metabolic product of many *Penicillium* species. It is widely used as an antifungal drug against dermatophytes. Cytochalasins²⁵ are fungal metabolites with a novel mechanism of action on mammalian cells. Many studies have shown that cytochalasins have potential for clinical use as anticancer agents⁶⁹. In conclusion, it should be stated that only a few examples of fungi uses have been mentioned. One can say that more investigations on fungi need to be done.

1.7 Natural products from endophytic fungi

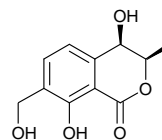
The secondary metabolites isolated from fungal endophytes have different biosynthetic pathways. They belong to different structural categories: isocoumarines, quinones, alkaloids, and phenols, *etc.*

1.7.1 Isocoumarin derivatives

(*R*)-Mellein (**1**), an isocoumarin isolated from *Pezicula* sp.⁷⁰, is a powerful fungicidal, herbicidal. Gamahorin (**2**) is an isocoumarin obtained from stromata of *E. typhina* on *P. pratense*⁷¹.



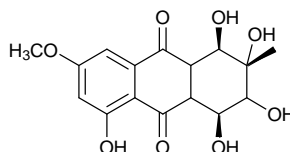
(1)



(2)

1.7.2 Quinones

Altersolanol A (**3**), is a highly hydroxylated quinone, found in phytopathogenic *Alternaria* cultures, was re-isolated from an endophytic *Phoma* sp. and found to be an antibacterial agent⁷².

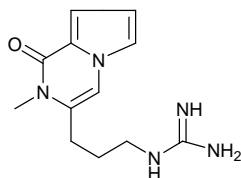


(3)

1.7.3 Alkaloids

Alkaloids are quite common secondary metabolites from endophytes, and some of them show antimicrobial activity. Amines and amides were isolated from *Acremonium* endophytes which was later re-classified as the genus *Neotyphodium*⁴⁸.

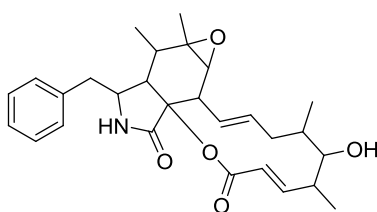
Peramine (**4**), a pyrrolopyrazine alkaloid, was extracted from *Neotyphodium coenophialum*, and from *E. typhina*⁷³. This secondary metabolite showed toxicity to insects with no harmful effect on mammals^{74, 75}.



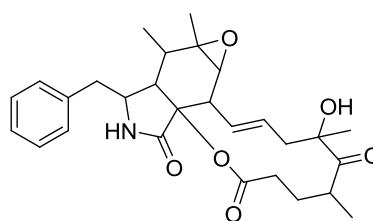
(4)

Another group of compounds that possess antitumor and antibiotic activities are cytochalasins. Cytochalasins are alkaloids which are commonly produced by endophytic fungi such as *Xylaria*, *Phoma*, *Hypoxylon* and *Chalara* genera.

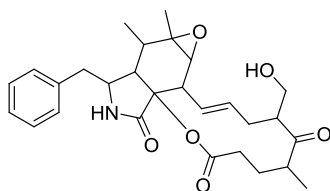
Furthermore, three new cytotoxic cytochalasins (**5-7**), together with the known metabolite cytochalasin E (**8**) were characterised from a *Rhinocladiella*⁷⁶ sp. commonly found as a saprophyte on dead tree limbs of a medicinal plant, *Tripterygium wilfordii*.



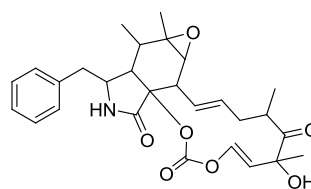
(5)



(6)



(7)

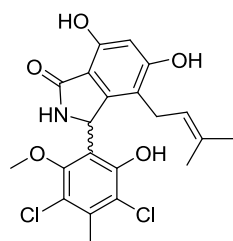
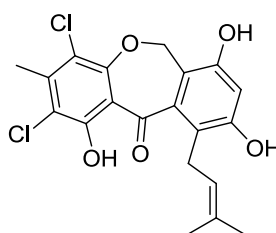
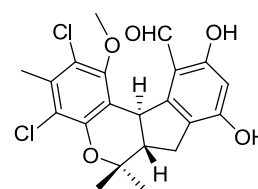


(8)

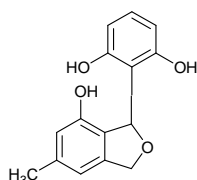
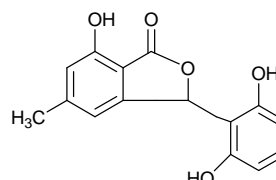
1.7.4 Phenols

Phenol and phenolic acids have often been isolated from endophyte cultures originating from a variety of host plants⁷⁷.

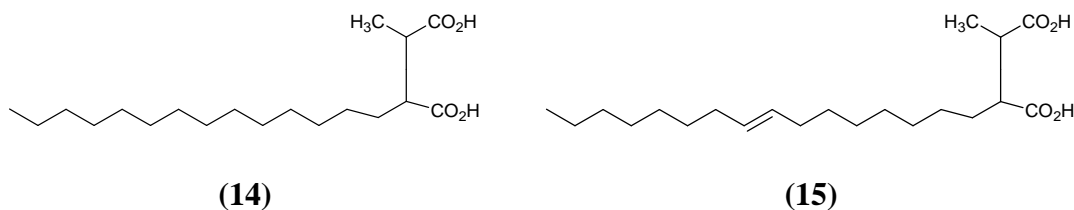
Pestalachloride A-C (**9-11**), three new chlorinated benzophenone derivatives, have been isolated from cultures of an endophytic fungus *Pestalotiopsis adusta*. Pestachlorides A and B displayed significant antifungal activities against three plant pathogens⁷⁸.

**(9)****(10)****(11)**

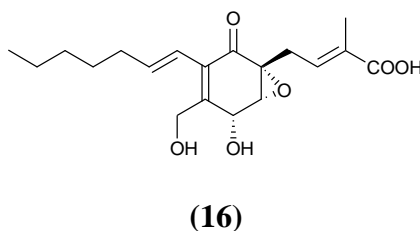
Endophytic fungi can produce compounds with antimicrobial as well as antioxidant activity. Pestacin (**12**) and isopestacin (**13**), have been isolated from culture media of *P. microspora*, an endophyte isolated from a plant called *Terminalia morobensis* growing in the Sepik river drainage of Papua New Guinea^{79, 80}. Both pestacin (**12**) and isopestacin (**13**) display antimicrobial as well as antioxidant activity^{79, 80}.

**(12)****(13)**

Chaetomelic acids A (**14**) and B (**15**) isolated from the culture of an endophytic *Chaetomella acutisea* were found to be specific inhibitors of farnesylprotein transferase (FPTase)⁸¹.



Ambuic acid (**16**), a highly functionalised cyclohexenone isolated from *P. microspora*, possesses antifungal activity⁸².



1.8 The aims of the project

This project is designed to achieve the following objectives:

- Create secondary metabolite profiles of the studied *Xylariaceous* and endophytic fungi.
- Search for novel and bioactive secondary metabolites produced by *Xylariaceous* and endophytic fungi.
- Apply NMR and other spectroscopic techniques to elucidate the structure of isolated secondary metabolites.

Chapter 2 Secondary metabolites from endophytic fungi

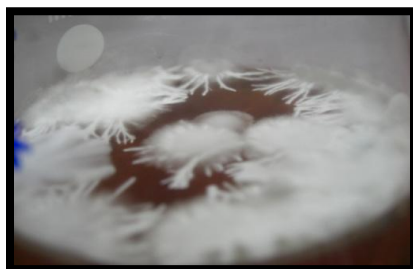
2.1 Endophytic fungi profile

Five endophytic fungi were collected from the palm tree leaves *Borassodendron machadonis* from Trad Province in Southern Thailand **Figure 1**.



Figure 1 *Borassodendron machadonis* palm tree

The fungi were provided by Prof. A-J-S Whalley, Liverpool John Moores University, as cultures in Petri dishes. The five endophytes, coded **B231b**, **B127R**, **B115**, **B338** and **A116**, were static sub-cultured for two weeks in ten conical flasks, two for each fungus. Each conical flask contained an aqueous malt extract medium. This Chapter describes the examination of cultures of the fungi for their metabolites. Fungi **B231b** and **B127R** grew with a broad white mycelium, whereas fungus **B115** developed light brown fruiting bodies, which grew 1.0-1.5 cm high with a base diameter of 0.5 mm on the white upper surface of the mycelium. Fungus **A116** developed black cylindrical shaped-stromata with a black base on a white mycelium, whilst fungus **B338** produced a black mycelium **Figure 2**.

**Fungus B231b****Fungus B127R****Fungus B115****Fungus A116****Fungus B338****Figure 2 Endophytic fungi after 10 days**

Each different endophyte was then transferred into 10 Thompson bottles (2 L) and allowed to grow on a sterile aqueous malt extract-glucose mixture for 8 weeks. Master cultures of each of these endophytes were made for future reference. The matured cultures were harvested and the mycelia removed by filtration through a muslin cloth.

The respective aqueous filtrates were extracted in batches with ethyl acetate in a separating funnel (5 L) and the combined ethyl acetate extracts for each fungus were dried over anhydrous sodium sulphate. Removal of the solvent by rotary evaporation gave the crude extracts details of which are summarised in **Table 3**.

Table 3 Summary of properties of endophytes crude extracts

Fungus	Crude Yields	Description
B127R	10.1 g	Brown gum
B338	4.6 g	Gummy brown liquid
B231b	6.2 g	Brown gum
B115	2.7 g	Brown gum
A116	0.58 g	Yellow crystals

Fungus **A116** gave yellowish crystals (0.58 g), which were triturated with acetone and recrystallised from methanol to give pure white hairy needles (12 mg) of cytochalasin D. TLC studies of the four crude extracts indicated that the endophytes (**B127R**, **B338**, **B231b** and **B115**) all produced the same metabolites.

2.2 Isolation of secondary metabolites from fungus B127R medium

2.2.1 Isolation of Cytochalasin D

The crude extract of **B127R** (10.1 g) was taken up into ethyl acetate (20 ml) producing a thick creamy solution. The solution set aside for 24 h. A fine white solid (106 mg)

precipitated from the solution. The solid was filtered off and recrystallised from methanol to give white needles (79 mg), mp 230-232 °C, ES $[M+H]^+$ m/z 508, $[\alpha]_D^{25}$ -12.5° (c 1, in dioxane). This compound showed an opaque spot when treated with diazotised *p*-nitroaniline spray reagent, at R_f 0.24 in solvent system; toluene, ethyl acetate and acetic acid (50:49:1) on TLC. The IR_{ATR} ν_{max} cm^{-1} spectrum of this compound showed signals at: 3419, 1741 and 1692, which could indicate the presence of the following functional groups: a hydroxyl, a carbonyl ester and an amide.

1H NMR spectrum (C_5D_5N , Figure 5) was complex showing several sets of peaks across the region δ 0.5 to δ 9.5. The ^{13}C NMR and DEPT-135 spectra (C_5D_5N , Figure 6&7) showed 28 carbon signals. The signals at δ_c 128.79 and 129.89 are double the height of other peaks signifying that these signals are each probably due to two carbon atoms giving a total of 30 carbons. The DEPT-135 spectrum showed four methyl signals at δ_c 13.59, 19.34, 20.51 and 24.60, three methylene signals at δ_c 38.54, 45.49, and 112.16 and seven quaternary signals at δ_c 210.86, 175.09, 170.45, 151.61, 138.43, 78.38 and 53.98 and seven methine signals in the olefinic aromatic/region between δ_c 126-134 and nine further methine carbon signals in the region of δ_c 13-80.

The spectral and physical data of this compound suggested it was a cytochalasin, which have been previously reported as secondary metabolites from *Xylariaceae* fungi^{28, 35}. Comparison of 1H and ^{13}C NMR data of this compound with the reported data²⁵ for a series of cytochalasins revealed that this compound was cytochalasin D ($C_{30}H_{37}NO_6$). The proton signal observed at δ_H 9.08 and the methylene signal in the DEPT spectrum at δ_c 112.16 are particularly indicative of cytochalasin D **Figure 3**.

The ^1H NMR, ^{13}C NMR and DEPT-135 assignments for cytochalasin D are presented in **Table 5**. 2D NMR techniques (^1H - ^1H COSY, HMQC and HMBC experiments) were used to determine connectivities in addition to the ^1H and ^{13}C NMR data.

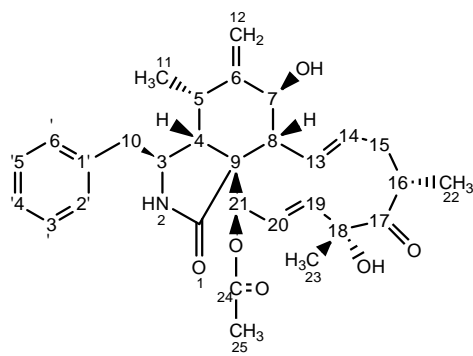


Figure 3 Cytochalasin D

^1H NMR spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 5) showed the presence of four methyl peaks in the δ_{H} 0.76 to 2.41 region. The most deshielded signal is the methyl of the ester group C-25 at δ_{H} 2.41. The magnetically non-equivalent protons 15-Ha and 15-Hb gave multiplets at δ_{H} 1.90-1.93 and 2.58-2.63 respectively. The 4-H proton appeared at δ_{H} 2.42, whereas, 3-H appeared as singlet at δ_{H} 3.54. The 10-H protons are represented by a multiplet signal at δ_{H} 2.80-2.83. The 7-H proton is seen at δ_{H} 4.40 and the hydroxyl proton at the same position appeared at δ_{H} 4.98 as a singlet, whilst the signals at δ_{H} 5.04 and 5.40 indicated possible alkene protons. The 13-H appeared as multiplet at δ_{H} 5.60-5.61, whilst 14-H gave a multiplet signal at δ_{H} 6.30-6.35. The other macrocyclic double bond has a multiplet signal at δ_{H} 5.54-5.59 for 19-H and doublet of doublet at δ_{H} 6.83 for 20-H. The 21-H proton appeared as a singlet at δ_{H} 6.18. The aromatic protons were grouped together at δ_{H} 7.20-7.35 and the compound specific N-H peak appeared downfield at δ_{H} 9.08.

The chemical shift of the N-H proton of the lactam ring in cytochalasin compounds appears generally above δ_H 9.0, when pyridine- d_5 is used as the NMR solvent. It has been observed that the signal for this proton can be characteristic for cytochalasin⁶⁹

Table 4. In cytochalasin D for instance, the N-H proton resonates at δ_H 9.08, which is the case for the isolated cytochalasin.

Table 4 Characteristic physical data of some cytochalasins²⁵

Cytochalasin	Amide δ_H in pyridine	mp °C	m/z
C	9.38	260- 265	507
D	9.13	267-270	507
N	9.55	272	523
O	9.25	258- 265	525
P	9.10	169-173	525
Q	9.45	145-147	507
R	9.7	159-167	523

The cytochalasins are a class of secondary metabolites produced during fungal growth, which exhibit unusual behaviour on animal cells. The series of structurally related natural products of ever increasing number has grown steadily in size since they were first isolated in the 1960s⁶⁹. The cytochalasins are generally characterised as containing a highly substituted perhydroisoindolone ring system which is fused to a macrocyclic ring of varying size (11-14 atoms in length and containing either carboxylic, lactonic or carbonate moieties), a typical example is cytochalasin C⁸³ **Figure 4.**

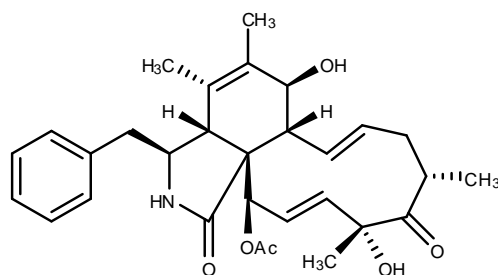


Figure 4 Cytochalasin C

The first members of the series were discovered independently by two groups almost simultaneously; Tamm and Rothweiler⁸³ working at the University of Basel, Switzerland, and Aldridge and Turner of ICI in Cheshire, UK⁸⁴. Since then it has been isolated by several other workers. Edwards *et al*²⁵ isolated this compound alongside other new cytochalasins N, O, Q, R and P from the fungus *Hypoxylon terricola* at the University of Bradford.

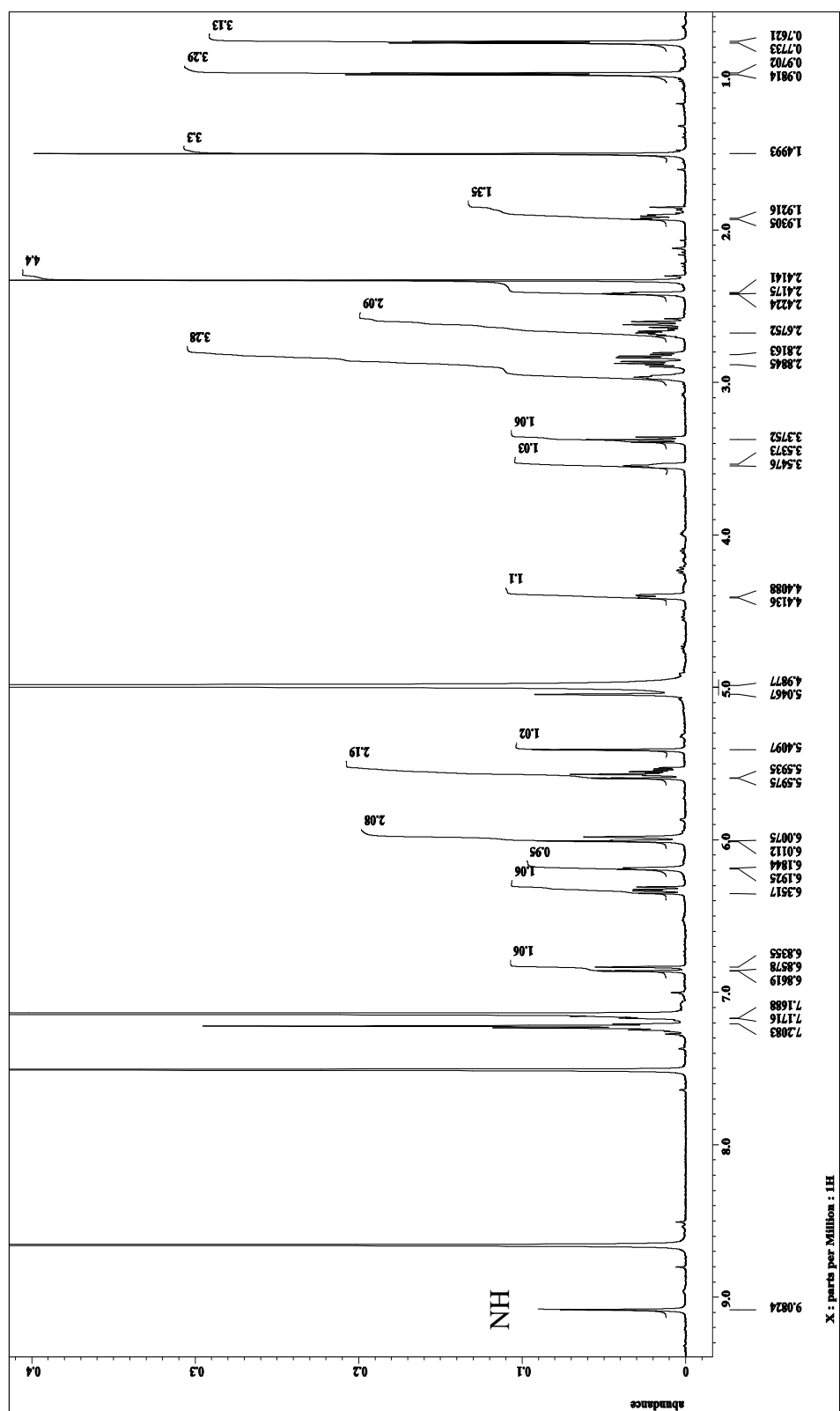
The name cytochalasin (from the Greek – *cytos*, a cell and – *chalysis*, relaxation) was proposed for this class of compounds, which was being descriptive of the characteristic effects when cells were treated with these compounds⁶⁹.

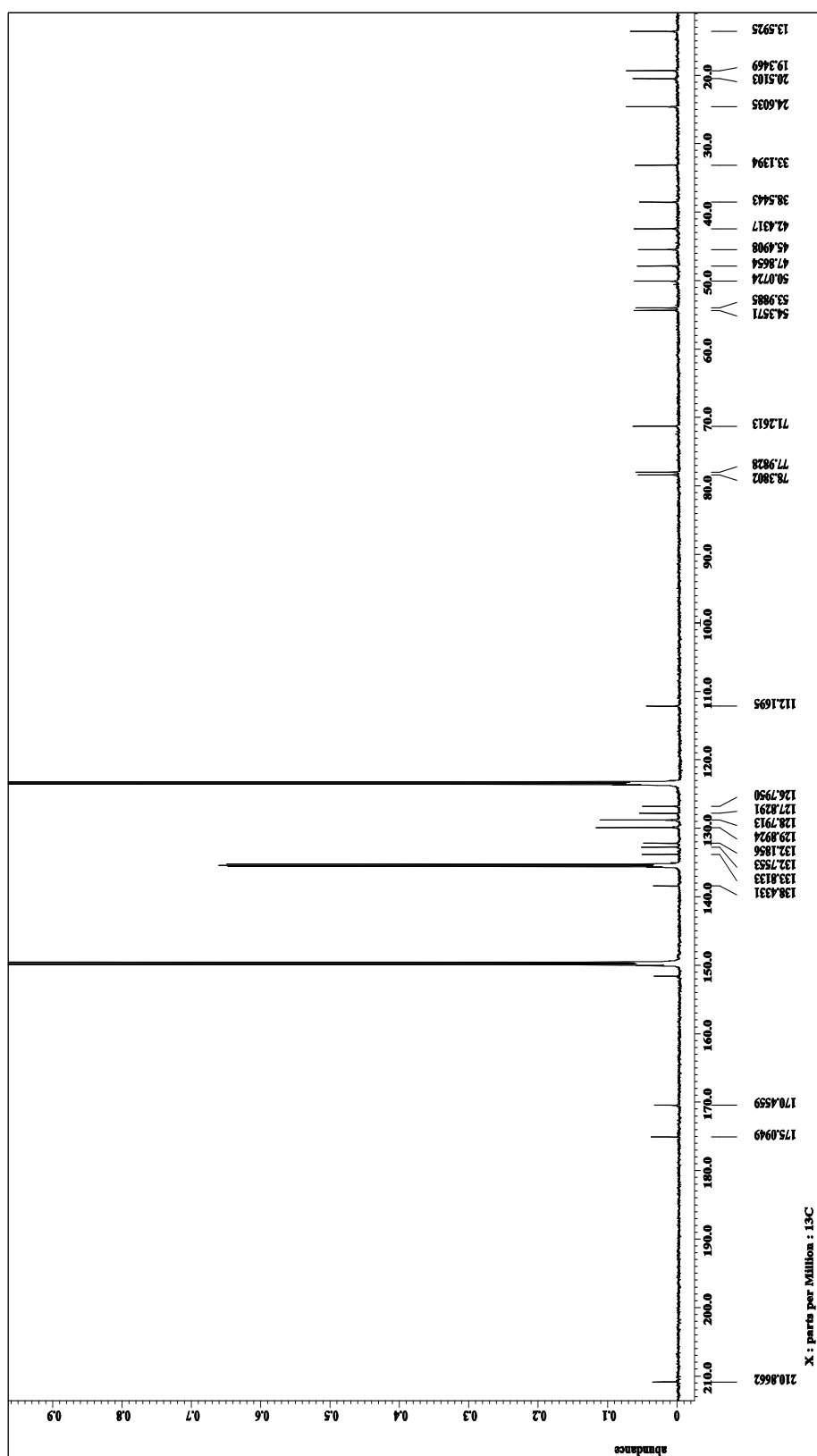
The biological activity of the cytochalasins was first reported by Carter⁶⁹ in the late 1960s, when unusual effects were observed upon specific strain of mouse fibroblast, which were treated with cytochalasin containing solution. It was discovered that cytochalasin B, at a concentration of 1µg/ml, prevented cytokinesis (cytoplasmic cleavage), whilst having no effect upon mitosis (nuclear division). Cytochalasin C and D were found to completely prevent cell cleavage at a dose as low as 0.05µg/ml. Under normal cellular conditions, mitosis occurs followed by the formation of a deep furrow in the cytoplasm, which cleaves to yield two identical cells. With the addition of

cytochalasin B to the culture medium, the treated cells undergo nuclear division as normal, but the cytoplasmic furrow does not fully develop and subsequently recedes. As a consequence, cytokinesis does not take place and leads to the formation of binucleated cells. Inhibition of cell motility was also observed at a concentration of 0.5µg/ml, when the addition of cytochalasin B to the culture medium immobilised cells for extended period of time, which was fully reversible with replacement of the old medium with fresh stock.⁶⁹ Cytochalasin D is a major metabolite produced by endophytic fungi which could be a source of a commercial supply of this metabolite.

Table 5 ^1H and ^{13}C NMR assignments for cytochalasin D ($\text{C}_5\text{D}_5\text{N}$)

No	δ_{H}	δ_{C}	DEPT
1		175.09	C
2	9.08 (1H, s)	NH	
3	3.54 (1H, s)	54.35	CH
4	2.42 (1H, s)	50.07	CH
5	3.00 (1H, s)	33.13	CH
6		151.61	C
7	4.40 (1H, d, J 10.3 Hz)	71.26	CH
7-OH	4.98 (1H, s)		
8	3.35-3.39 (1H, m)	47.86	CH
9		53.98	C
10	2.80-2.83 (2H, m)	45.49	CH_2
11	0.76 (3H, d, J 6.7 Hz)	13.59	CH_3
12a	5.04 (1H, s)	112.16	CH_2
12b	5.40 (1H, s)		
13	5.60-5.61 (1H, m)	127.82	CH
14	6.30-6.35 (1H, m)	132.75	CH
15a	1.90-1.93 (1H, m)	38.54	CH_2
15b	2.58-2.63 (1H, m)		
16	2.66-2.70 (1H, m)	42.43	CH
17		210.86	C
18		78.38	C
19	5.54-5.59 (1H, m)	132.18	CH
20	6.83 (1H, dd, J 2.5, 13.3 Hz)	133.81	CH
21	6.18 (1H, s)	77.98	CH
22	0.97 (3H, d, J 6.5 Hz)	19.34	CH_3
23	1.49 (3H, s)	20.51	CH_3
24		170.45	C
25	2.41 (3H, s)	24.60	CH_3
1'		138.43	C
2',6'	7.20-7.35 (2H, m)	129.89	CH
3',5'	7.20-7.35 (2H, m)	128.79	CH
4'	7.20-7.26 (1H, m)	126.79	CH

Figure 5 ^1H NMR spectrum of cytochalasin D

Figure 6 ^{13}C NMR spectrum of cytochalasin D

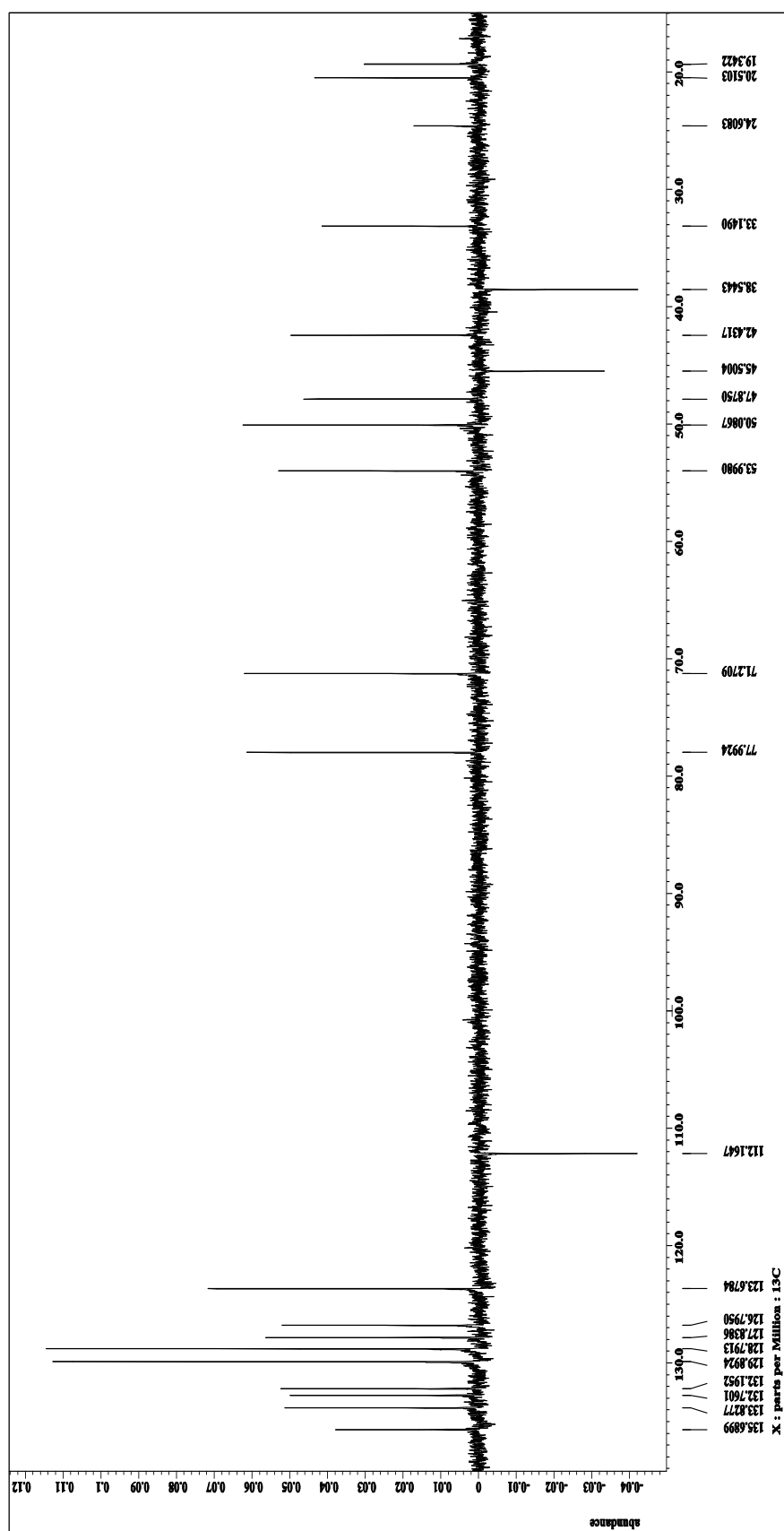
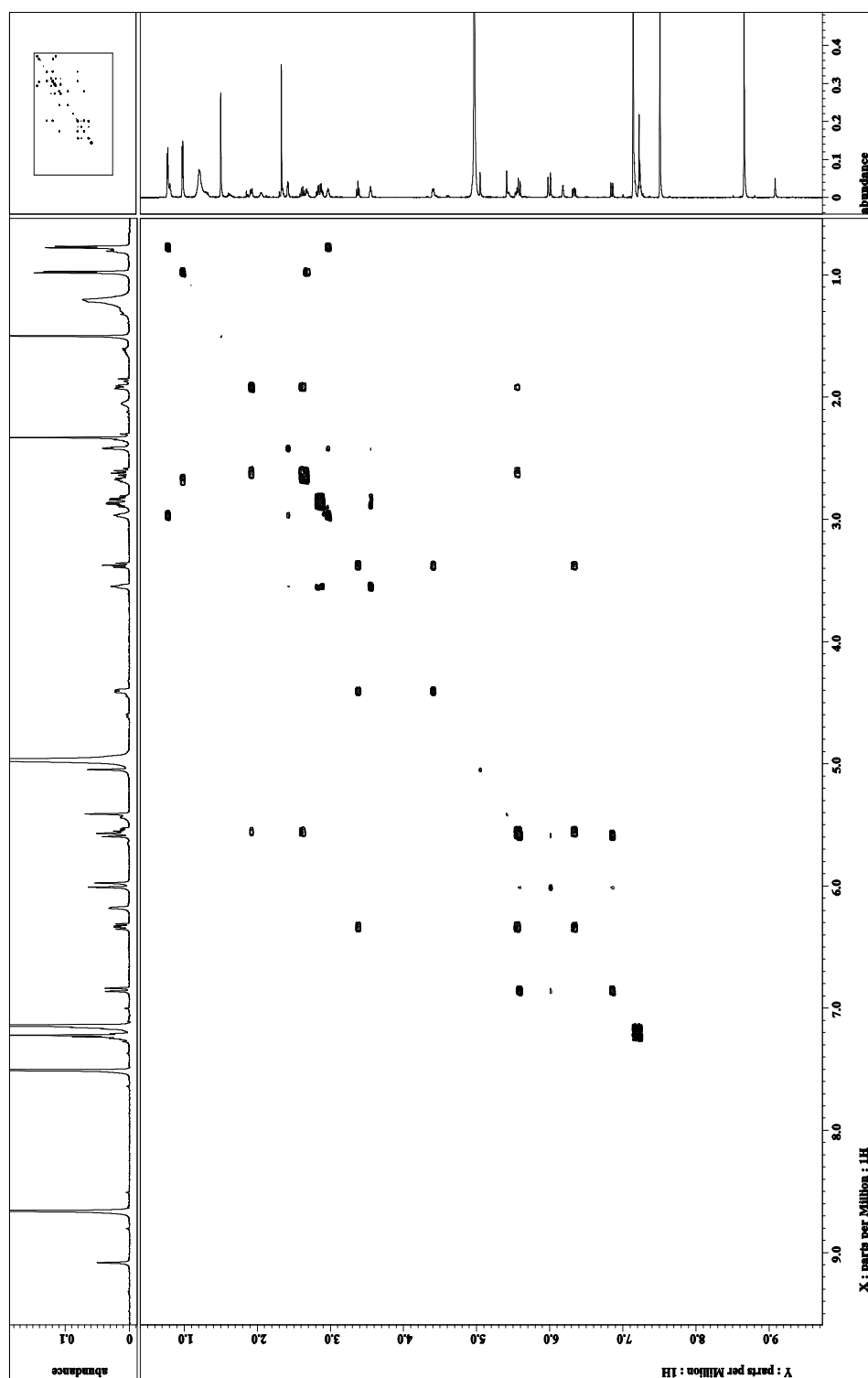


Figure 7 DEPT-135 NMR spectrum of cytochalasin D

Figure 8 ^1H - ^1H COSY NMR spectrum of cytochalasin D

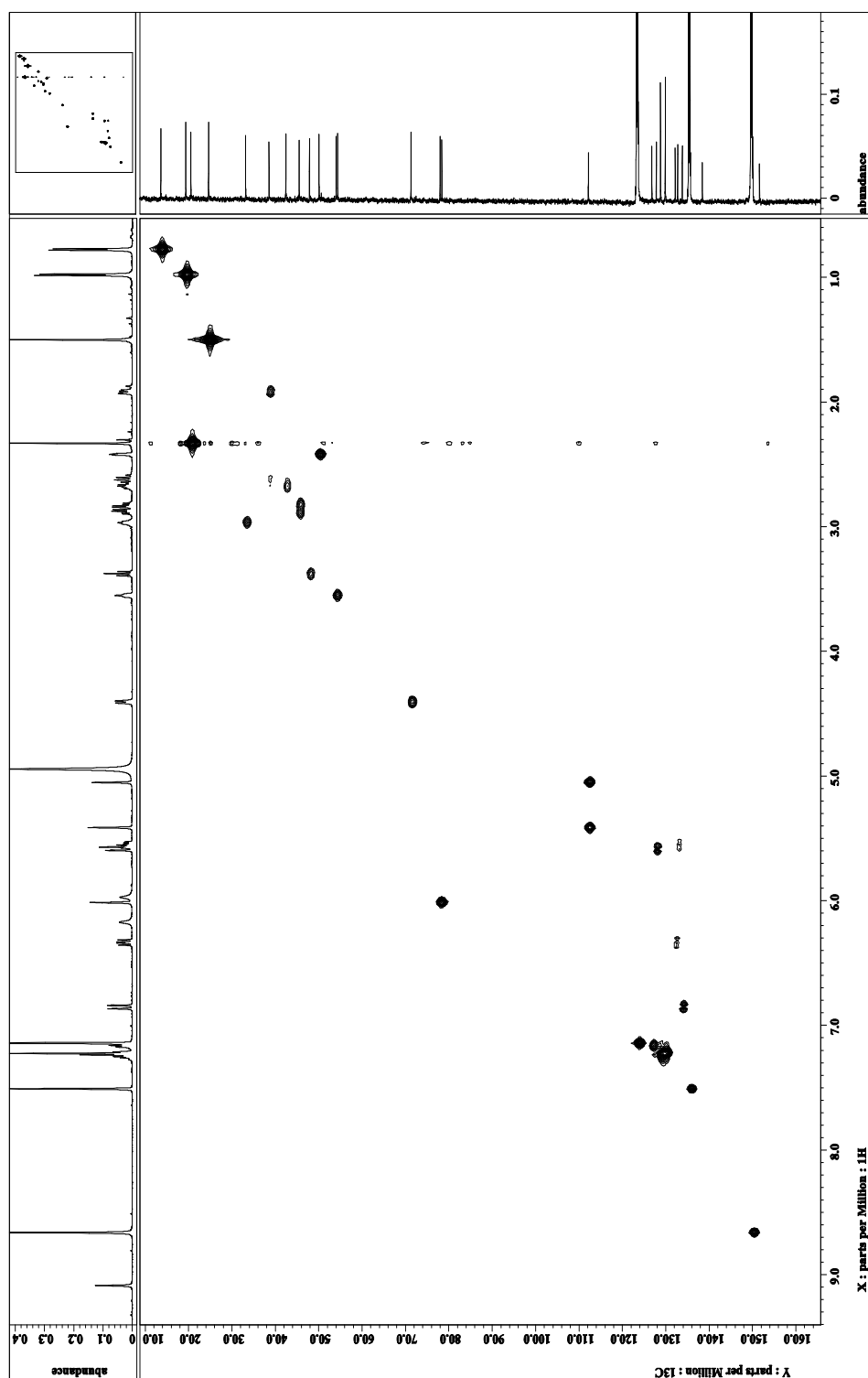


Figure 9 HMQC NMR spectrum of cytochalasin D

2.2.2 Isolation of Coriloxin

After the isolation of cytochalasin D, the remaining filtrate was concentrated *in vacuo* to yield a brownish gummy liquid (5.0 g), which was triturated with acetone and set aside overnight. This afforded dark brown crystals, which were recrystallised twice from toluene to give optically active colourless needles (103 mg), mp 153-155, °C, ES $[M+H]^+$ m/z 171, $[\alpha]$ -98.4° (c 1, in EtOH), IR_{ATR} ν_{\max} cm⁻¹ 3403, 1648 and 1607 cm⁻¹. The strong peak at 3403 cm⁻¹ suggests the presence of a hydroxyl group. Two bands at 1648 and 1607 cm⁻¹ might be due to a conjugated carbonyl and a double bond. The ¹H NMR spectrum (CDCl₃, Figure 11) showed only 6 sets of signals indicative of ten protons. The signal at δ_H 5.23 is most likely a methine proton attached to an olefinic carbon. A low field methine proton at δ_H 4.46 might be due to an attachment to an oxygen to the carbon bearing this proton. The resonance position of the methoxy group at δ_H 3.73 suggests it is attached to an unsaturated carbon. There is a singlet at δ_H 1.62 indicative of a methyl group, which could possibly be attached to a carbon directly attached to an oxygen atom. The resonance peak at δ_H 2.68 is due to a hydroxyl proton which is indicated in the IR spectrum.

The ¹³C NMR spectrum (CDCl₃, Figure 12) showed eight carbon resonances. A DEPT-135 spectrum showed these comprised two methyl signals at δ_C 19.02 and 56.69, three methine signals at δ_C 60.65, 69.21 and 98.29 and three quaternary carbons at δ_C 59.55, 171.54 and 193.56. The resonance peak at δ_C 193.56 is strongly indicative of a conjugated ketone, which is supported by an IR absorption at 1648 cm⁻¹. The signals at δ_C 56.69, 59.55, 60.65 and 69.21 suggested carbons adjacent to oxygen atoms. Excluding the carbonyl signal at δ_C 193.56, three oxygen atoms are left for four carbons, which suggests the presence of an epoxide group. The physical and spectral data of this compound was compared with literature data⁸⁵, which

revealed that this compound is coriloxin **Figure 10**. A summary of ^1H NMR, ^{13}C NMR and DEPT- 135 data are listed in **Table 6**.

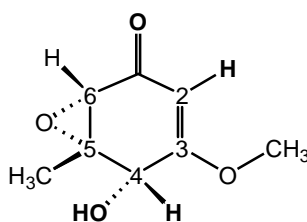
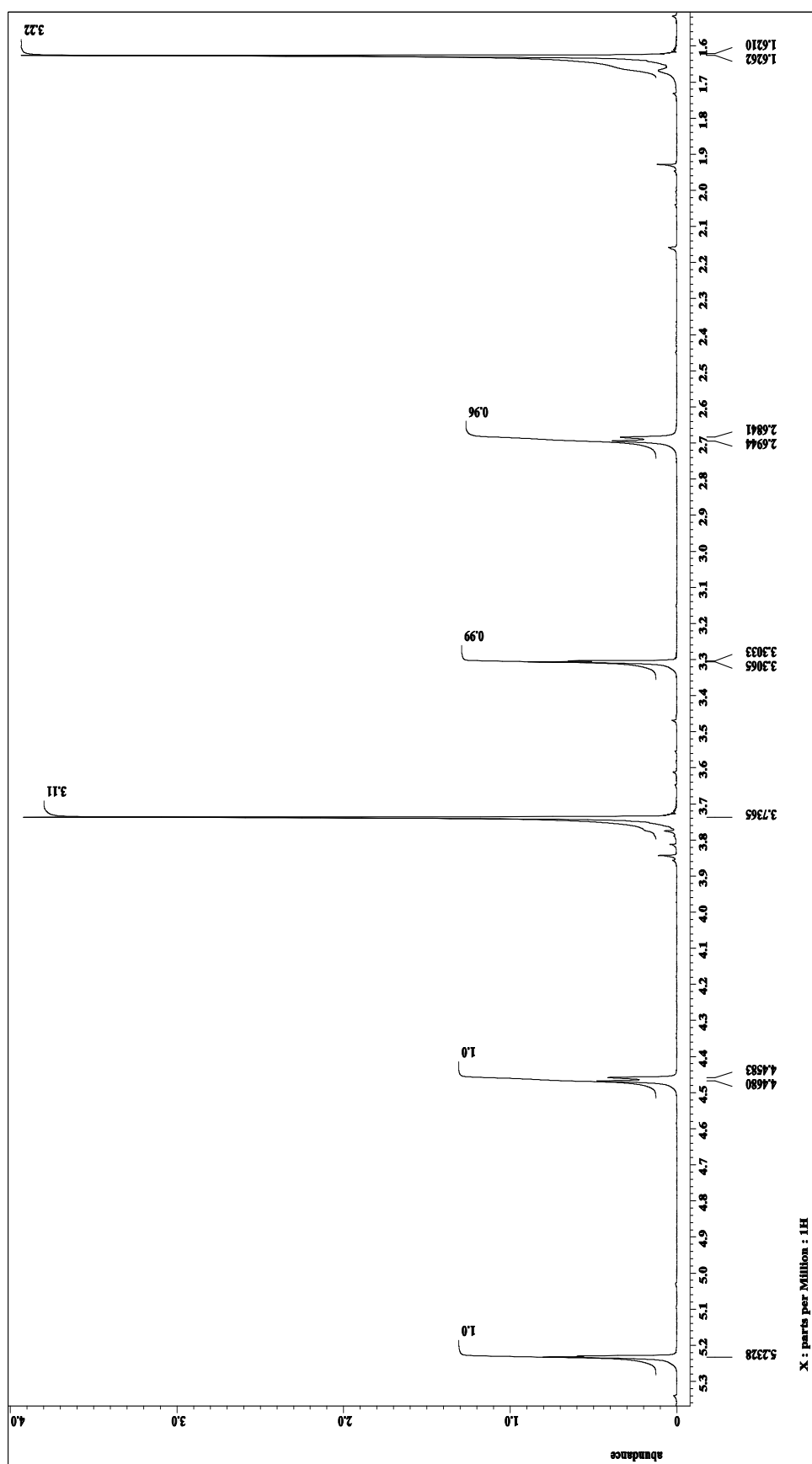


Figure 10 Coriloxin

Table 6 ^1H and ^{13}C NMR data of coriloxin (CDCl_3)

No	δ_{C}	Multiplicity	δ_{H}
1	193.56	C	
2	98.29	CH	5.23 (1H, d, J 1.7 Hz)
3	171.54	C	
4	69.21	CH	4.46 (1H, d, J 6.2 Hz)
5	59.55	C	
6	60.65	CH	3.30 (1H, d, J 1.7 Hz)
7	19.02	CH_3	1.62 (3H, s)
8	56.69	CH_3	3.73 (3H, s)
*	OH group	OH	2.68 (1H, d, J 6.2 Hz)

Coriloxin is a known natural product. It was first isolated and identified from the culture medium of the fungus *Coriolus vernicipes* in Japan⁸⁵. It was also reported from unpolished rice fermented with a *Xylariaceous* endophytic fungus³⁶. It shows antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* with MIC values of 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$ respectively³⁶. Coriloxin has been isolated previously at University of Bradford from *X. Obovata*⁸⁶, *X. Badia*³⁷, Adeboya⁸⁶ and other *Xylaria* species⁸⁷.

Figure 11 ^1H NMR spectrum of coriloxin

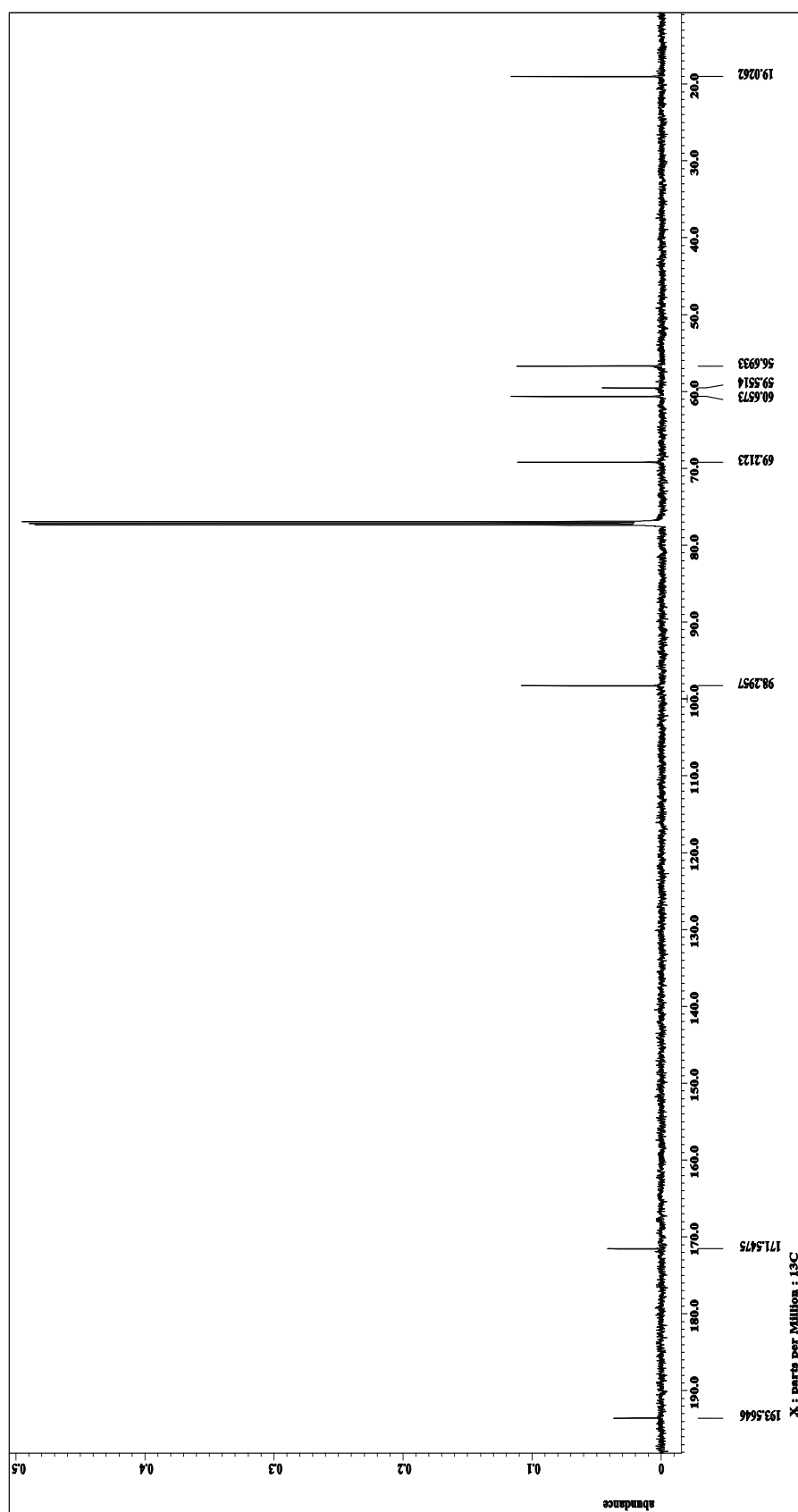
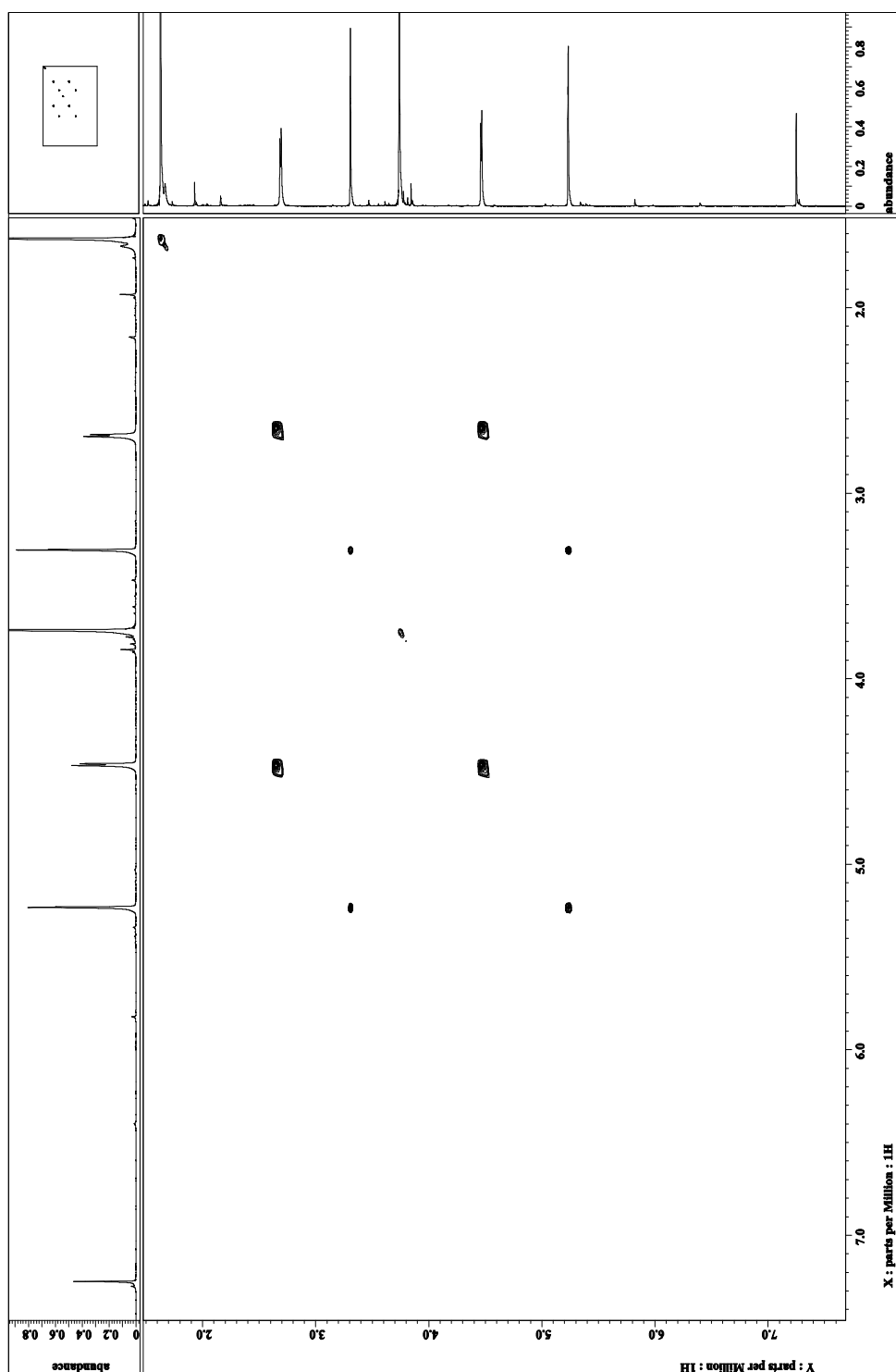


Figure 12 ^{13}C NMR spectrum of coriloxin

Figure 13 ^{13}C - ^1H COSY NMR spectrum of coriloxin

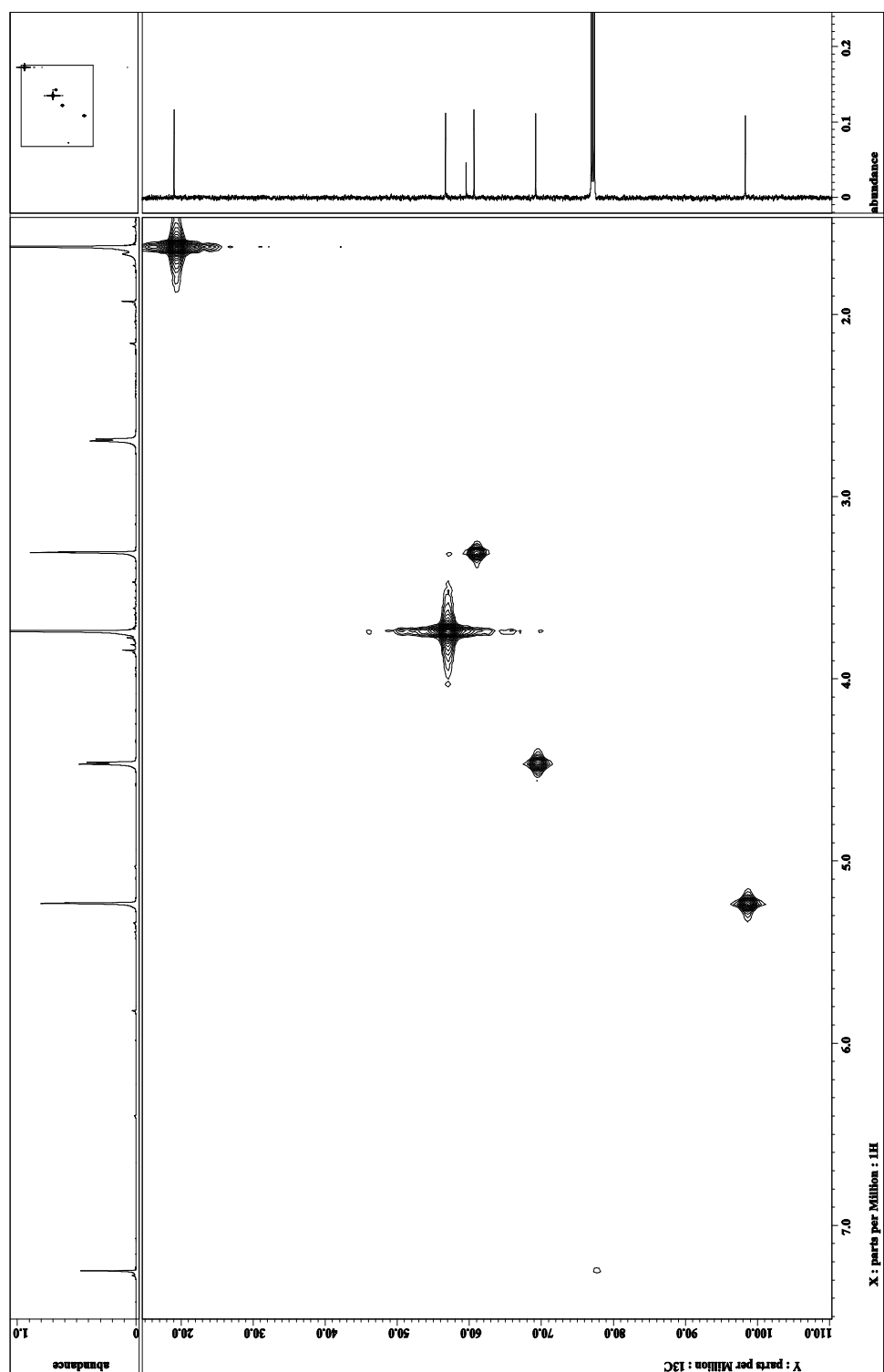


Figure 14 HMQC NMR spectrum of coriloxin

2.2.3 Isolation of Mellein

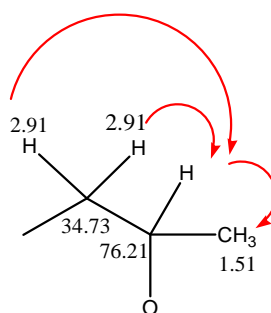
After removal of coriloxin the crude product had a dry weight of (3.3 g). Two portions (1 g each) of this brownish viscous liquid were chromatographed on two large thick layer plates (20 x 80 cm); (1.0 g) on each plate. The plates were developed in a tank containing a solvent system toluene, ethyl acetate, acetic acid (500:490:10). After the plates developed, they were removed and air-dried and then strips (1.0 cm) at the edges were sprayed with diazotised *p*-nitroaniline spray reagent to detect the bands.

(Band 1) (R_f 0.91) was scraped off and washed with ethyl acetate which was removed *in vacuo* to yield a yellow oil, which was triturated with *n*-hexane to give a powdery solid (7.5 mg) which was recrystallised from the same solvent to yield colourless plates (3 mg), mp 52-53 °C, ES $[M+H]^+ m/z$ 179, $[\alpha]_D^{25} +87^\circ$ (c 1, in $CHCl_3$), $IR_{ATR} \nu_{max} cm^{-1}$ 3187, 1662. The IR absorptions suggested the presence of hydroxyl and carbonyl groups. The compound had R_f 0.91 on a silica gel TLC plate eluted with toluene, ethyl acetate, acetic acid (50:49:1). It showed a strong orange spot with diazotised *p*-nitroaniline; suggesting the compound possessed a phenolic group, but was not active with anisaldehyde spray reagent.

The 1H NMR spectrum ($CDCl_3$, Figure 17) had a strong singlet at δ_H 11.02 indicating the presence of a chelated OH. Three signals were observed in the aromatic region (δ_H 6.5-8.5); two doublets at δ_H 6.67 (J 7.3 Hz) and 6.87 (J 8.4 Hz) coupled to a pseudo triplet at δ_H 7.39 (J 7.5 Hz). These signals indicated a tri-substituted ring. Also, three signals at δ_H 1.51 (3H, d, J 6.3 Hz), 2.91 (2H, d, J 7.0 Hz) and a multiplet signal in the region 4.70-4.73 (1H, m). The position of this methine proton indicates that it is attached to a C-O group.

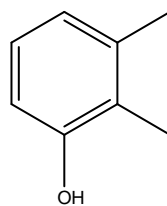
In the ^{13}C NMR spectrum (CDCl_3 , Figure 18) ten carbon signals comprise a methyl carbon at δ_{C} 20.88, a methylene carbon at δ_{C} 34.73, a methine at δ_{C} 76.21 and a carbonyl at δ_{C} 170.07. Six resonance positions are in the aromatic regions which occur at δ_{C} 108.41, 116.36, 118.00, 136.25, 139.50 and 162.31. The quaternary carbon at δ_{C} 170.07 is a typical of a carbonyl of a lactone. The chelated OH occurs at δ_{H} 11.02 indicating that the OH is in the neighbourhood of the carbonyl group according to the low infrared-absorption at 1662 cm^{-1} . The mass spectrum, ^1H and ^{13}C NMR suggested a molecular formula of $\text{C}_{10}\text{H}_{10}\text{O}_3$.

In the ^1H - ^1H COSY spectrum (CDCl_3 , Figure 20) the doublet methyl proton at δ_{H} 1.51, which correlates to δ_{C} 20.88 in the HMQC spectrum (CDCl_3 , Figure 21) showed coupling to a methine proton at δ_{H} 4.70-4.73, which correlates to δ_{C} 76.21 in the HMQC. The down field carbon signal at δ_{C} 76.21 suggested an adjacent oxygen atom. The methine proton at δ_{H} 4.70-4.73 also coupled to the methylene protons at δ_{H} 2.91 which showed a cross peak with δ_{C} 34.73 in the HMQC spectrum. This give a partial structure X.



X

The three protons in the aromatic region at δ_{H} 6.67, 6.87 and 7.39 suggested a phenolic ring. Also, six aromatic carbons three of which are quaternary according to the DEPT-135 analysis, (CDCl_3 , Figure 19) suggesting a 1,2,3-trisubstituted benzene ring; partial structure Y.



Y

The singlet proton at δ_{H} 11.02 is a characteristic signal for a chelated–OH group. The carbon signal at δ_{C} 170.07 suggested an ester carbonyl carbon. According to the low infrared absorption at 1662 cm^{-1} a chelated lactone system was likely. Combining the two structures gave the structure of mellein **Figure 15**.

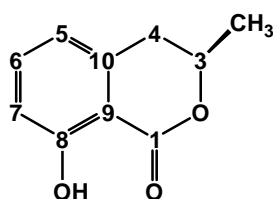


Figure 15 Structure of mellein

The NMR assignments for the compound, which were established using 1D and 2D NMR spectral experiments, ^1H , ^{13}C , DEPT-135, ^1H - ^1H COSY and HMQC experiments, are presented in **Table 7**.

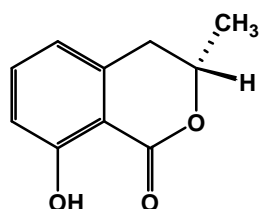
Table 7 ^1H and ^{13}C NMR assignments of mellein (CDCl_3)

No	δ_{C}	δ_{H}
1	170.07-C	
3	76.21-CH	4.70-4.73 (1H, m)
4	34.73-CH ₂	2.91 (2H, d, J 7.0 Hz)
5	118.00-CH	6.87 (1H, d, J 8.4 Hz)
6	136.25-CH	7.39 (1H, t, J 7.5 Hz)
7	116.36-CH	6.67 (1H, d, J 7.3 Hz)
8	162.31-C	
9	108.41-C	
10	139.50-C	
11	20.88-CH ₃	1.51 (3H, d, J 6.3 Hz)
*	OH group	11.02 (1H, s)

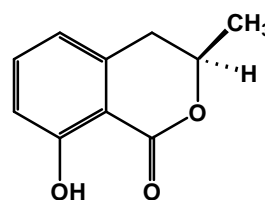
Mellein is a common secondary metabolite from *Xylaria* fungi. It was first isolated in 1933 from *Aspergillus melleus*^{39,88}. It has also been isolated in Bradford from different *Xylaria* fungi such as *X. Longiana*³³, *X. Grammica*³⁵, *X. Badia*³⁷ and other species of *Hypoxylon*⁸⁹. It has also been found^{39, 89} that fungi which produced this metabolite can produce other derivatives such as 4-hydroxymellein, 5-carboxymethylmellein and 8-*O*-methylmellein.

Mellein exists naturally in two enantiomer forms; the most common form *R*-(-)-mellein where optical rotation $[\alpha] -92.5^\circ$ (c 1, in EtOH) was isolated from *Lasiodiplodia theobromae*⁴¹. Islam *et al* reported⁹⁰ the synthesis of *R*-(-)-mellein with optical rotation $[\alpha] -102^\circ$ (c 0.53, in CHCl_3)⁹⁰. Dimitriadis *et al* reported⁹¹ the stereospecific synthesis of both enantiomers of mellein, where the optical rotation of

S-(+)-mellein is $[\alpha]_D^{25} +88.6^\circ$ (c 0.27, in MeOH) and for *R*-(-)-mellein is $[\alpha]_D^{25} -101.3^\circ$ (c .07, in CHCl₃). By comparing these optical rotations with that of the isolated mellein the later was confirmed as *S*-(+)-mellein **Figure 16**.

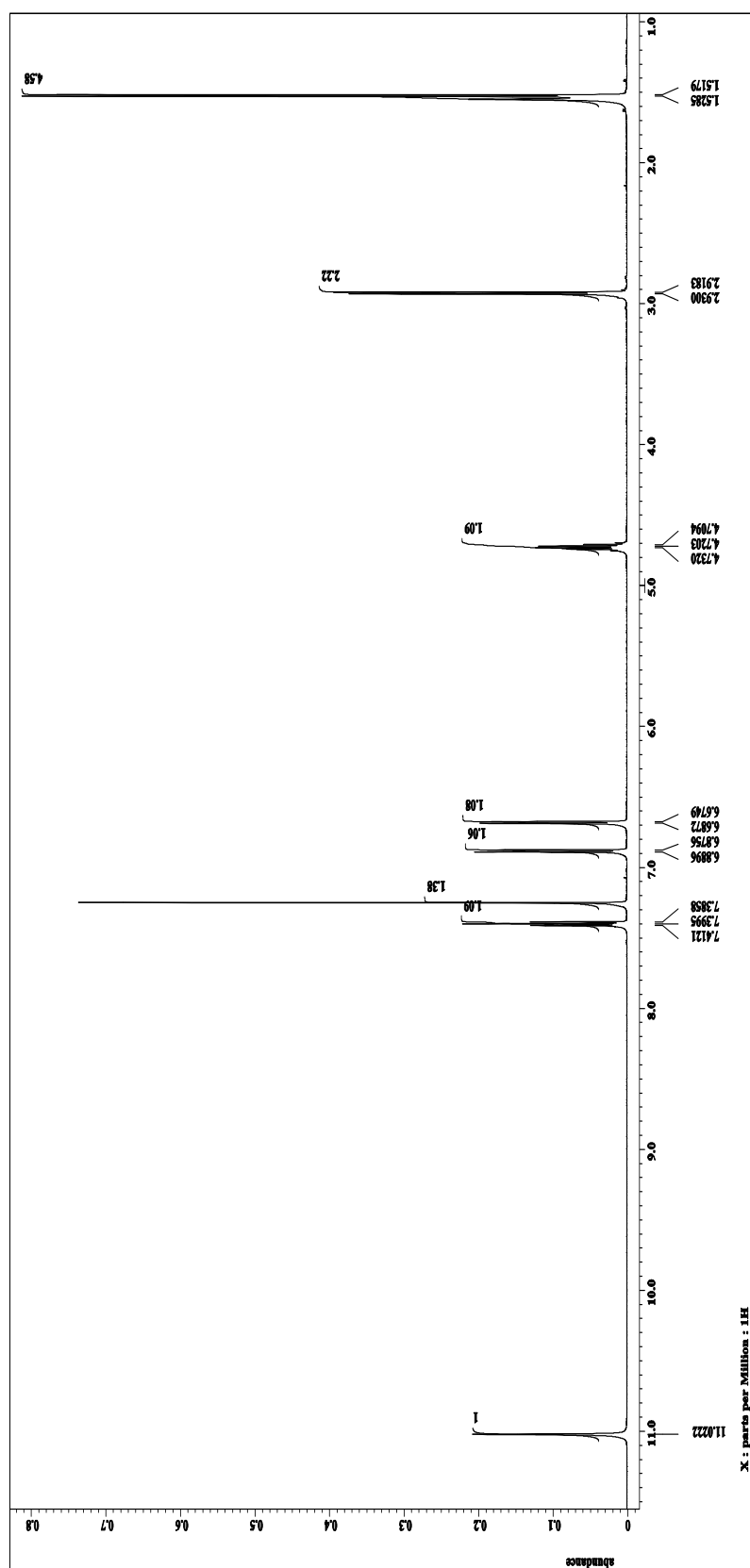


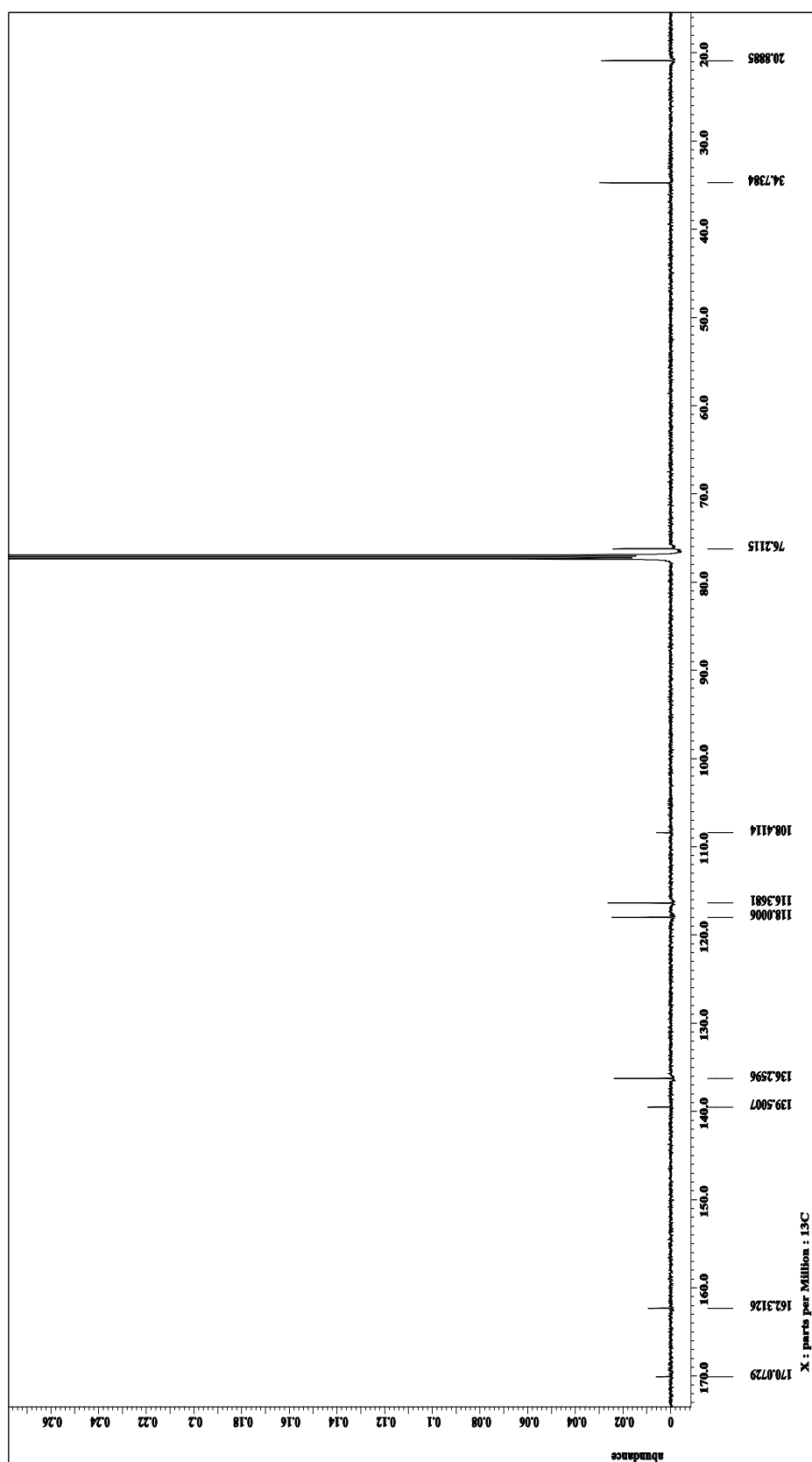
S-Mellein



R-Mellein

Figure 16 *S* and *R* enantiomers of mellein

Figure 17 ¹H NMR spectrum of mellein

Figure 18 ^{13}C NMR spectrum of mellein

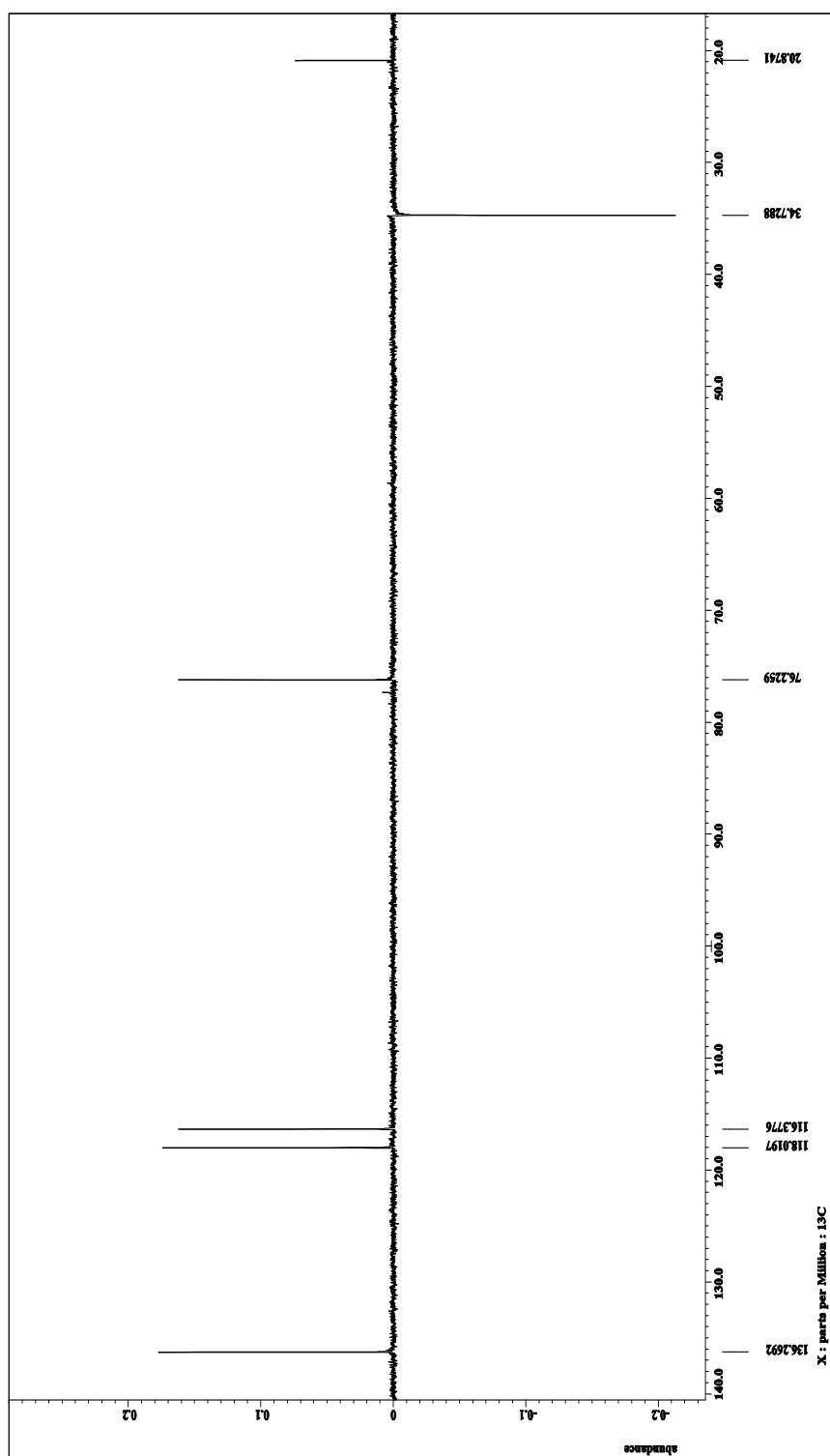
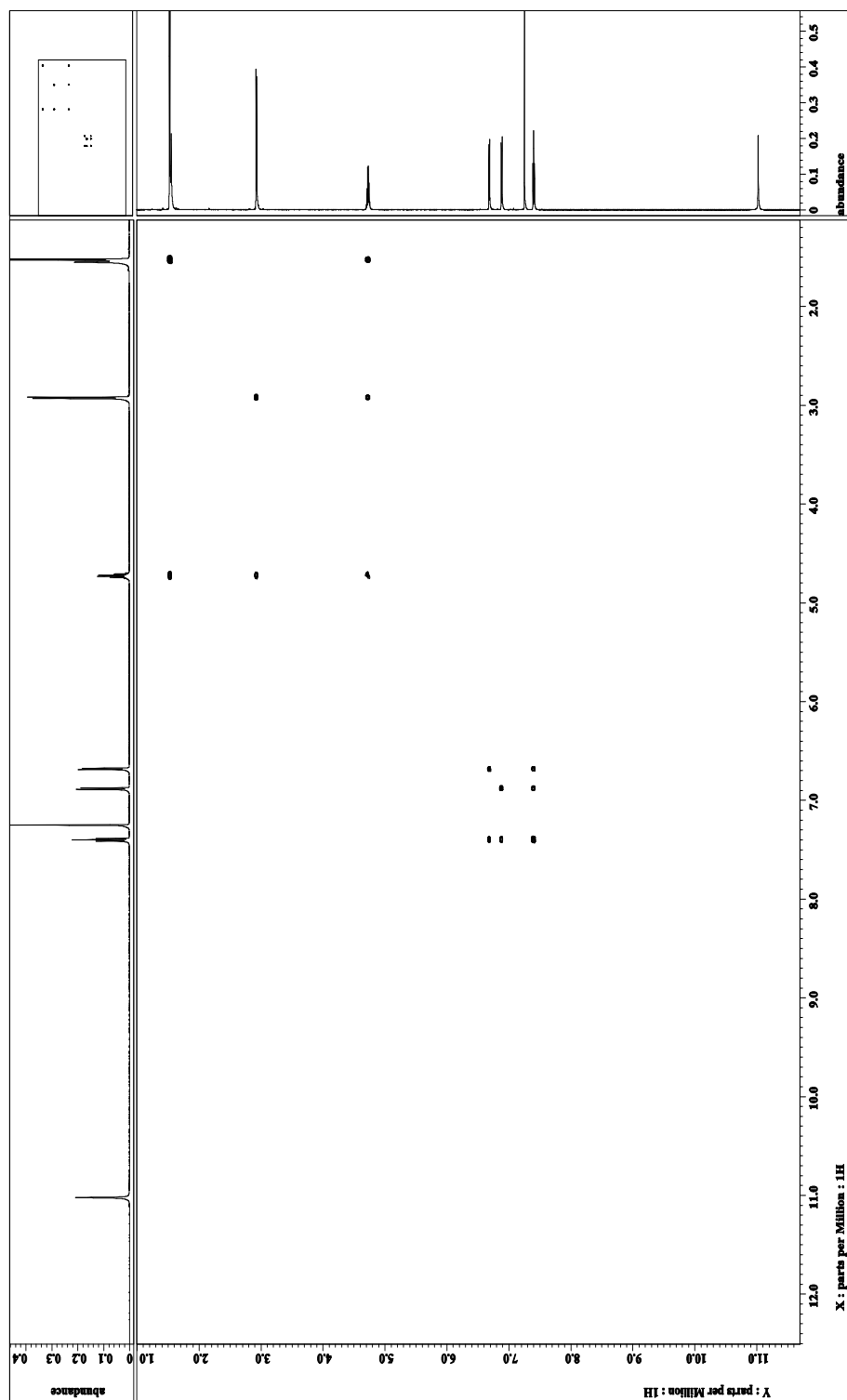


Figure 19 DEPT-135 NMR spectrum of mellein

Figure 20 ^1H - ^1H COSY NMR spectrum of mellein

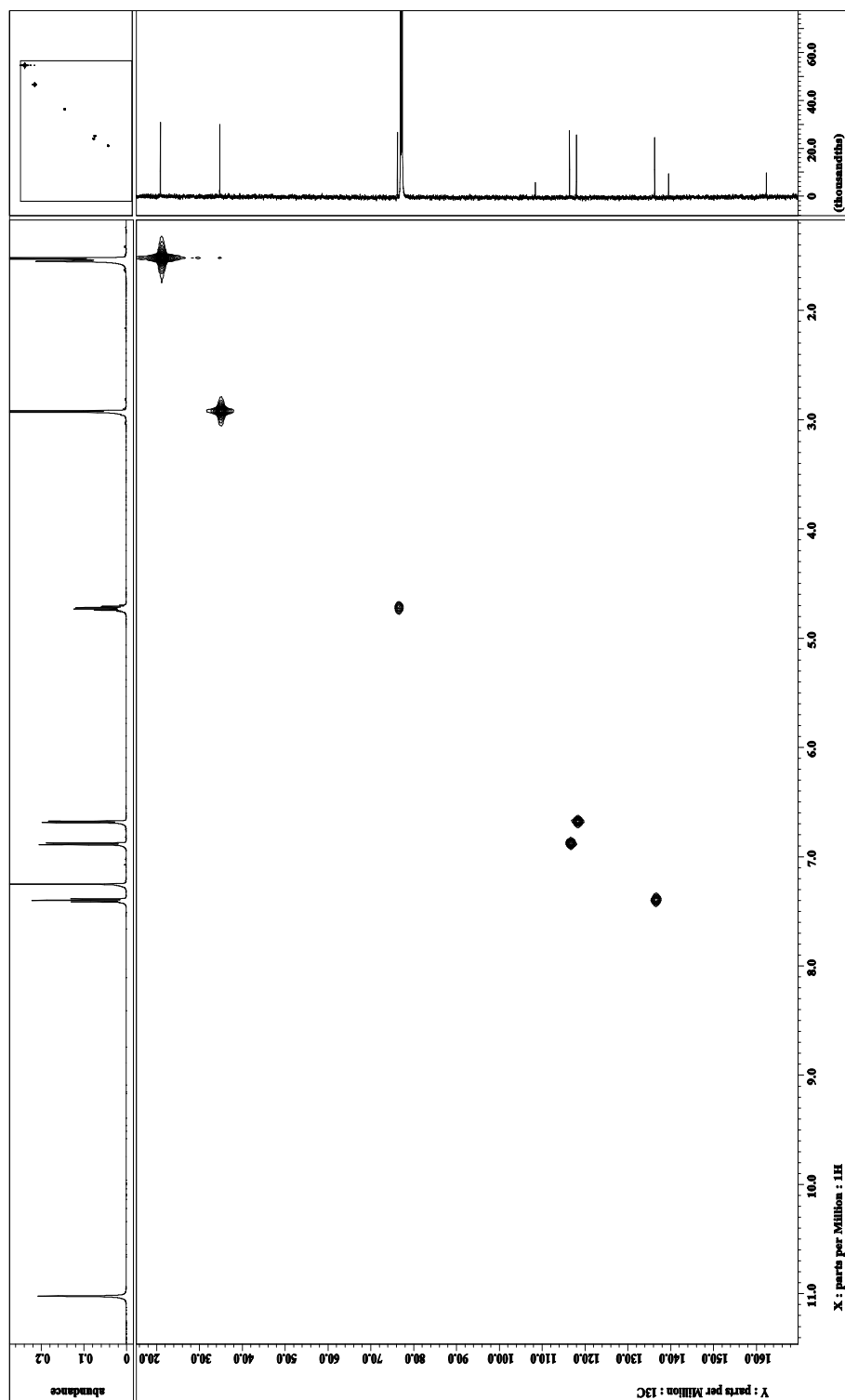


Figure 21 HMQC NMR spectrum of mellein

2.2.4 Isolation of *cis*-4-Hydroxymellein

The next UV active band on the preparative TLC plate at R_f 0.71, that developed a strong orange colouration when sprayed with diazotised *p*-nitroaniline spray reagent; suggesting a phenolic compound, was recovered. The recovered silica was washed with ethyl acetate to yield a yellow solution. The solvent was removed *in vacuo* to produce yellowish needles, which were recrystallised from toluene to yield fine colourless prisms, (87 mg), mp 112-117 °C, ES $[M+H]^+$ m/z 195, $[\alpha]_D^{25} +48^\circ$ (c 1, in MeOH), IR_{ATR} ν_{\max} cm⁻¹ 3430, 3178, 2997, 1642 and 1615. IR absorptions at 3430 and 3178 cm⁻¹ in the spectrum of this compound indicated the presence of two hydroxyl groups. The absorption at 3178 cm⁻¹ suggested a hydrogen bonded hydroxyl group, whilst the absorption at 3430 cm⁻¹ indicated a non-hydrogen bonded OH.

The ¹H NMR spectrum (CDCl₃, Figure 25) suggested a mellein type compound. The methylene signal of mellein at δ_H 2.91 is absent and has been replaced by an oxygenated methine proton at δ_H 4.68. The proton spectrum showed a singlet at δ_H 10.98 suggesting the presence of the chelated-hydroxyl group. Three peaks were observed in the aromatic region (δ_H 6.5-8.5); 6.90 (1H, d, J 7.3 Hz), 7.01 (1H, dd, J 8.4, 0.9 Hz) and 7.50 (1H, t, J 7.3 Hz). A methyl group at δ_H 1.57 (3H, d, J 6.6 Hz). A methine proton at δ_H 4.69 (1H, qd, J 6.6, 2.0 Hz) could be attached to an oxygenated carbon. A ¹H-¹H COSY spectrum (CDCl₃, Figure 28) showed coupling between the doublet of quartets of δ_H 4.69 and the doublet methyl group at δ_H 1.57 as well as the doublet methine proton at δ_H 2.04 (1H, d, J 6.4 Hz), which would account for the OH group at the C-4. The chelated-OH signal at δ_H 10.98 suggests that this group is in the neighbourhood to the quaternary carbon at δ_C 169.26 which is indicative for an ester carbonyl group. The IR spectrum shows a band at 1642 cm⁻¹,

characteristic of a hydrogen bonded lactone system. The ^1H - ^1H COSY spectrum indicated coupling between the aromatic protons. The proton at δ_{H} 6.90 (1H, d, J 7.3 Hz) and proton at δ_{H} 7.01 (1H, dd, J 8.4, 0.9 Hz) are coupled to a pseudo triplet at δ_{H} 7.50 (1H, t, J 7.3 Hz); indicative of a three proton spin- system. In the ^{13}C NMR spectrum (CDCl_3 , Figure 26) showed six aromatic carbons are detected at δ_{C} 162.25, 140.58, 136.92, 118.71, 118.39 and 106.94, four of which are detected as quaternary carbons according to the DEPT-135 (CDCl_3 , Figure 27); suggesting the ring is tri-substituted. The ^1H NMR has also detected the possible coupling between the hydroxyl peak at δ_{H} 2.04 (OH, d, J 6.4 Hz) and the methine peak at δ_{H} 4.68 (1H, dd, J 6.1, 1.8 Hz). By combining the m/z of mellein to that of this compound, it is 16 units larger than that of mellein, indicating additional oxygen. The absence of a methylene signal in the carbon spectrum of this compound compared to that of mellein indicates this compound is substituted at C-4.

The replacement of the methylene carbon in mellein with a down field carbon at δ_{C} 78.27 suggested the substitution at C-4 to be a hydroxyl group, which means that this compound is 4-hydroxymellein **Figure 22**. Comparison of the ^1H and ^{13}C NMR data of this compound with literature data⁹² showed it to be *cis*-4-hydroxymellein^{41, 92} (**Figure 23**; structures A and B).

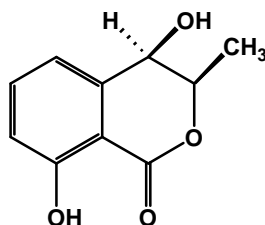


Figure 22 4-Hydroxymellein

It is possible to decide, if there is a *cis* or *trans* relationship between the protons at C-3 and C-4 in 4-hydroxymellein, by determination the coupling constant between the two protons. A coupling constant⁹³ of 2 Hz is consistent with the *cis* form, whilst a 4 Hz coupling constant is associated with the *trans* form.

A spin decoupling NMR experiment was performed by irradiation of the methyl group protons at position C-11. This eliminated the coupling between the methyl group protons and the proton at position C-3. The quartet of doublets signal at 4.69 Hz collapsed leaving a doublet peak. The *J*-coupling between the 3-H and 4-H was measured as 2.0 Hz. On this basis, the *cis*-configuration was assigned.

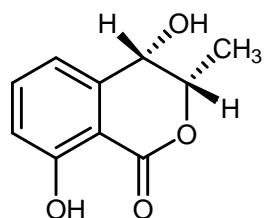
The NMR data of the compound were established by using 1D and 2D NMR spectral experiments; ¹H, ¹³C, COSY and HMQC. The later revealed all H-C direct correlations and thus confirmed the assignment of all carbons with attached protons

Table 8.

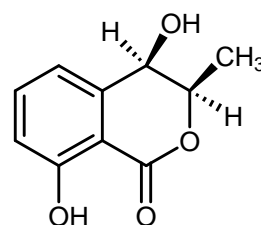
Table 8 ^1H and ^{13}C assignments for 4-hydroxymellein (CDCl_3)

No	δ_{C}	δ_{H}
1	169.26-C	
3	78.27-CH	4.69 (1H, qd, J 6.6, 2.0 Hz)
4	OH group	2.04 (1H, d, J 6.4 Hz)
4	67.39-CH	4.68 (1H, dd, J 6.1, 1.8 Hz)
5	118.71-CH	6.90 (1H, d, J 7.3 Hz)
6	136.92-CH	7.50 (1H, t, J 7.3 Hz)
7	118.39-CH	7.01 (1H, dd, J 8.4, 0.9 Hz)
8	162.25-C	
9	106.94-C	
10	140.58-C	
11	16.15-CH ₃	1.57 (3H, d, J 6.6 Hz)
*	OH group	10.98 (1H, s)

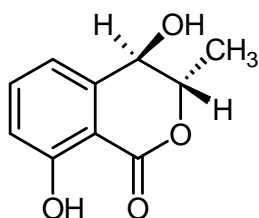
The 4-hydroxymellein molecule can exist in four stereoisomeric forms due to the chiral centres at C-3 and C-4 **Figure 23**. There are two *cis*-isomers (*3S,4S*)-4-hydroxymellein (**A**), (*3R,4R*)-4-hydroxymellein (**B**), and the two *trans*-isomers (*3S,4R*)-4-hydroxymellein (**C**), and (*3R,4S*)-4-hydroxymellein (**D**).



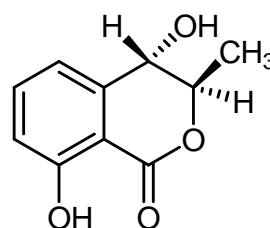
(*3S,4S*)-4-hydroxymellein (**A**)



(*3R,4R*)-4-hydroxymellein (**B**)



(*3S,4R*)-4-hydroxymellein (**C**)



(*3R,4S*)-4-hydroxymellein (**D**)

Figure 23 4-Hydroxymellein enantiomers

The compound can exist as one of two *cis*-isomers **Figure 23**. The relative stereochemistry of the compound was determined by x-ray diffraction studies⁹⁴. The data showed the hydrogen atoms at C-4 and C-3 were in the axial and equatorial positions respectively, confirming not only that the compound is in the *cis*-form, but it also showed that the compound is in the (*3R,4R*) configuration **Figure 24**.

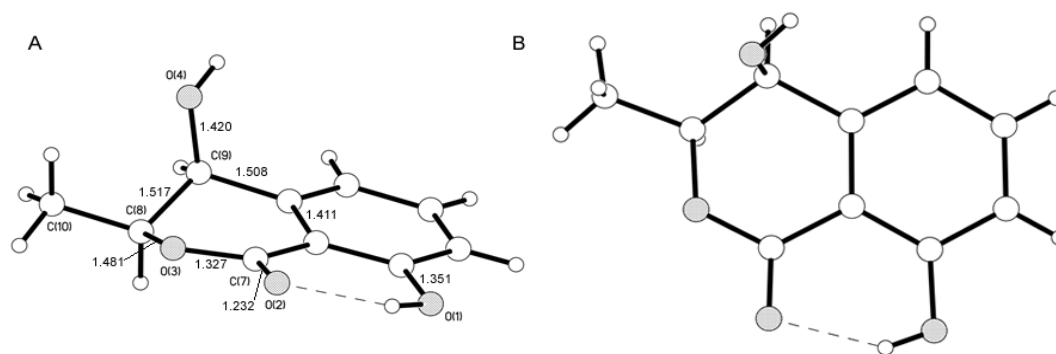


Figure 24 (3*R*,4*R*)-4-Hydroxymellein⁹⁴

4-Hydroxymellein of undetermined stereochemistry has been isolated previously from *Aspergillus oniki*⁹⁵, *Anisospora camptospora*⁴¹ and *Aspergillus ochraceus*⁴⁰. *Cis*-3*S*,4*S*-4-Hydroxymellein (**A**) in addition to mellein, has been isolated from the mycelium of *Cercospora taiwanensis*⁹⁶ grown on potato-agar. 3*R*,4*R*-4-Hydroxymellein has also been found in cultures from *Lasiodiplodia theobromae*⁴¹, *Septoria nodorum*⁹⁷ and *Aspergillus melleus*^{39, 92}.

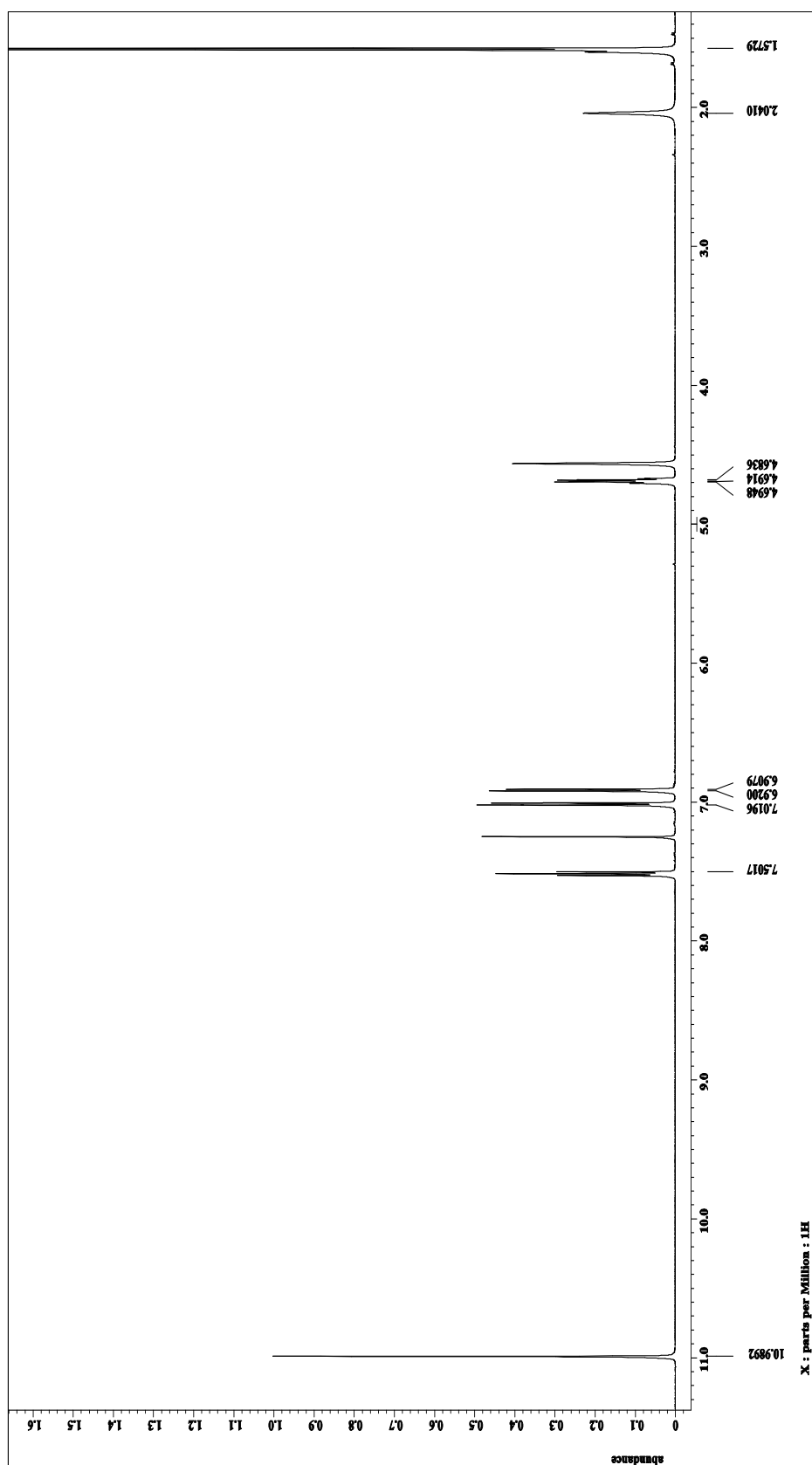
3*S*,4*R*-4-Hydroxymellein (**C**) has been isolated previously at Bradford University from the fungus *Xylaria grammica*³⁵. 3*R*,4*S*-4-Hydroxymellein (**D**) has been isolated from the medicinal plant *Moringa oleifera*⁹⁸ and together with the 3*R*,4*R* isomer from the fungus *Septoria*³⁸.

Cis-4-hydroxymellein has been tested for bioactivity^{47, 99}. It showed good activity against the alga *Chlorella fusca* and against the Gram positive *Bacillus megaterium*. It also showed antifungal activity against *Microbotryum violaceum*, which suggest that the endophytic fungus could protect the host by producing metabolites, which may be toxic or even lethal to phytopathogens and highlights the potential of endophytic fungi in producing bioactive metabolites^{47, 99}.

(3*R*,4*R*)-4-Hydroxymellein and (3*R*,4*S*)-4-hydroxymellein were both isolated from *Sephaeropsis sapinea*¹⁰⁰. They showed a synergistic activity, when assayed for phytotoxic and antifungal activities on host and non-host plants¹⁰⁰.

2.3 Isolation of cytochalasin D from fungus B127R mycelium

The air-dried mycelium was pulverised (53.9 g) and soxhlet extracted with chloroform (400 ml) for 16 h to give a brownish gum (7.6 g). The gum was dissolved in warm ethyl acetate (15 ml) and set aside for 24 h. A white solid (2.3 g) was obtained after the solution was filtered off. TLC studies showed that the solid was a cytochalasin since it showed an opaque spot after spraying with diazotized *p*-nitroaniline spray reagent. ¹H NMR and ¹³C NMR spectra (C₅D₅N) indicated that the compound was cytochalasin D, which was isolated and characterised from the culture medium of the same fungus.

Figure 25 ¹H NMR spectrum of *cis*-4-hydroxymellein

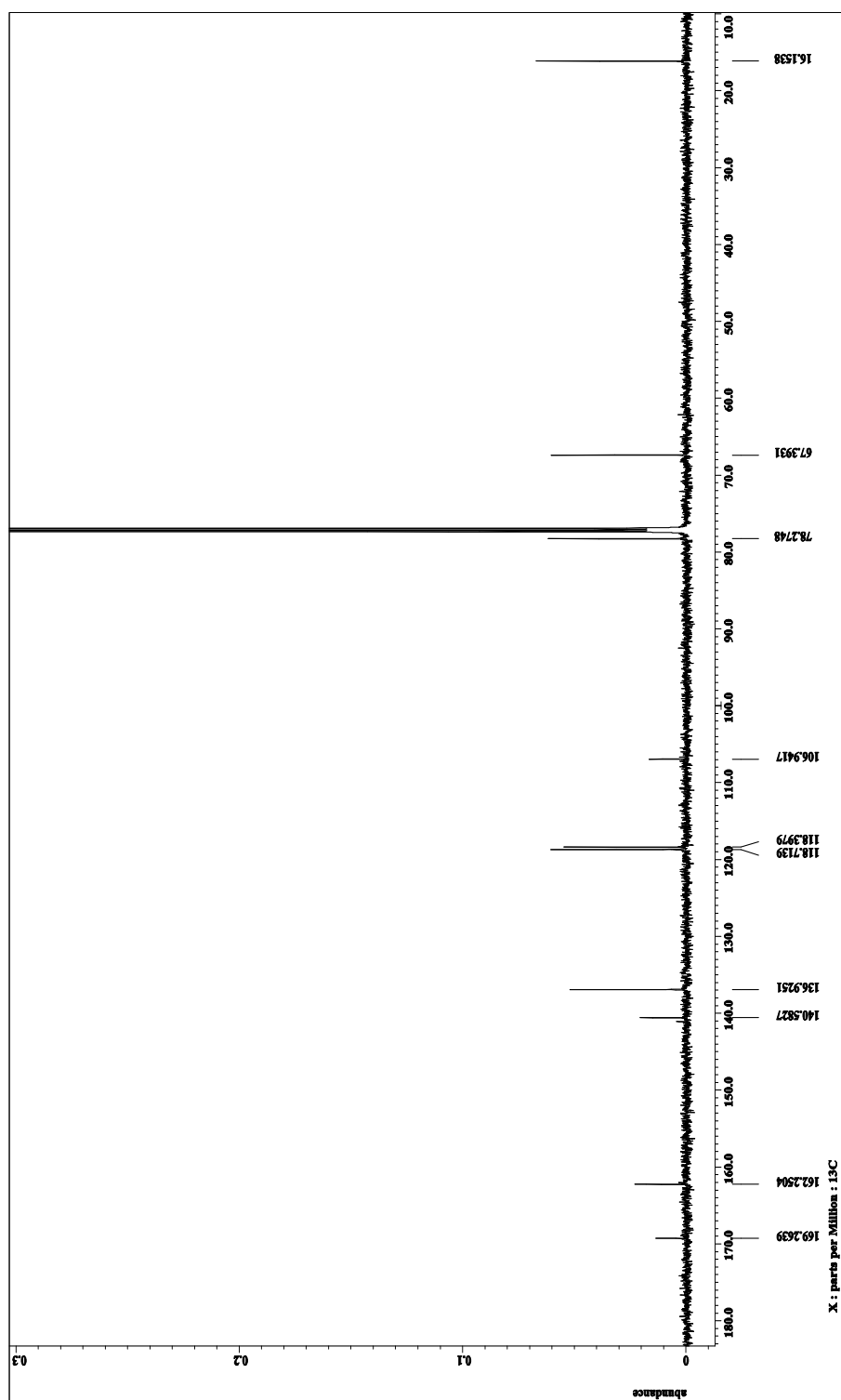
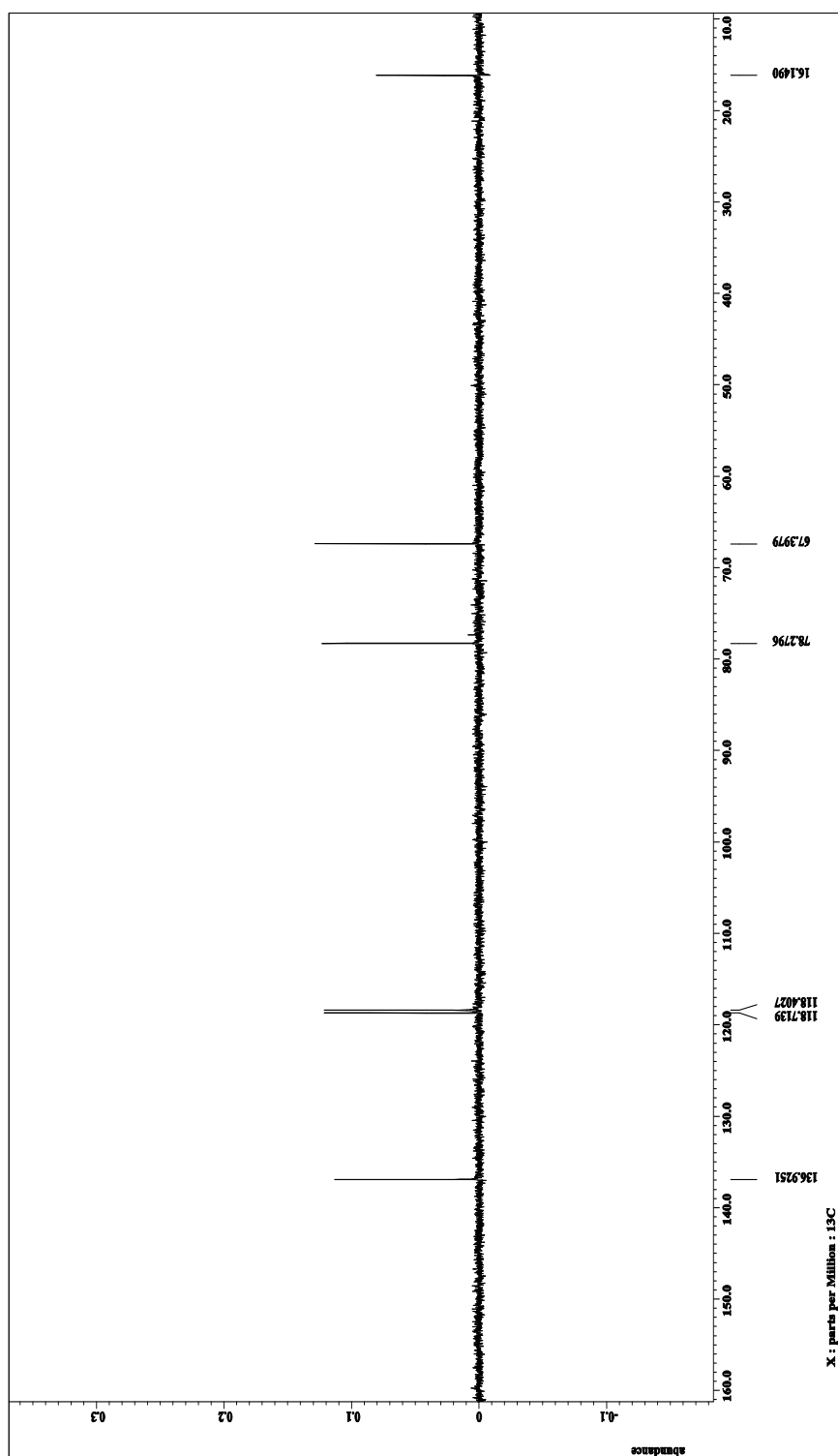


Figure 26 ^{13}C NMR spectrum of *cis*-4-hydroxymellein

Figure 27 DEPT-135 spectrum of *cis*-4-hydroxymellein

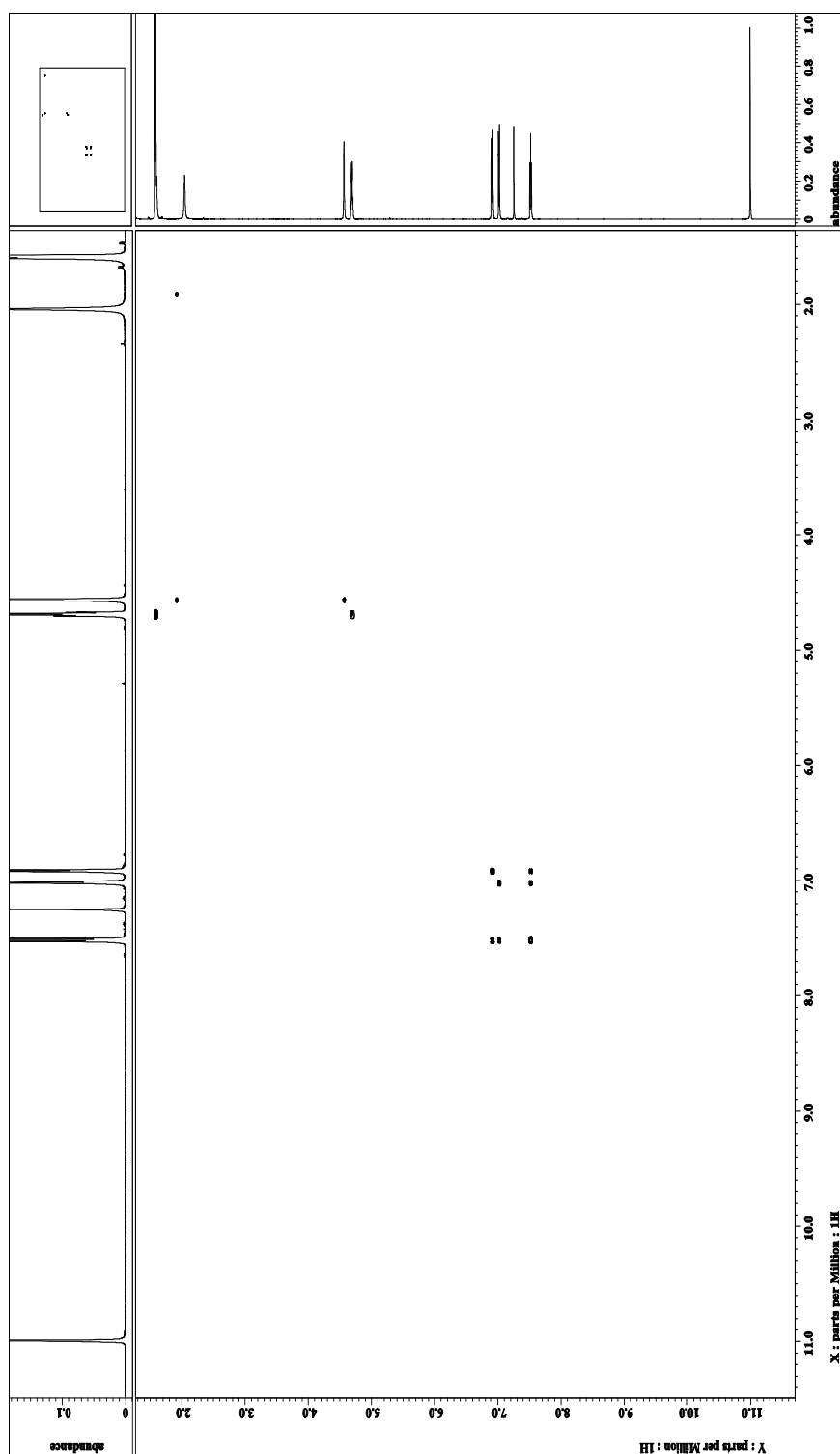
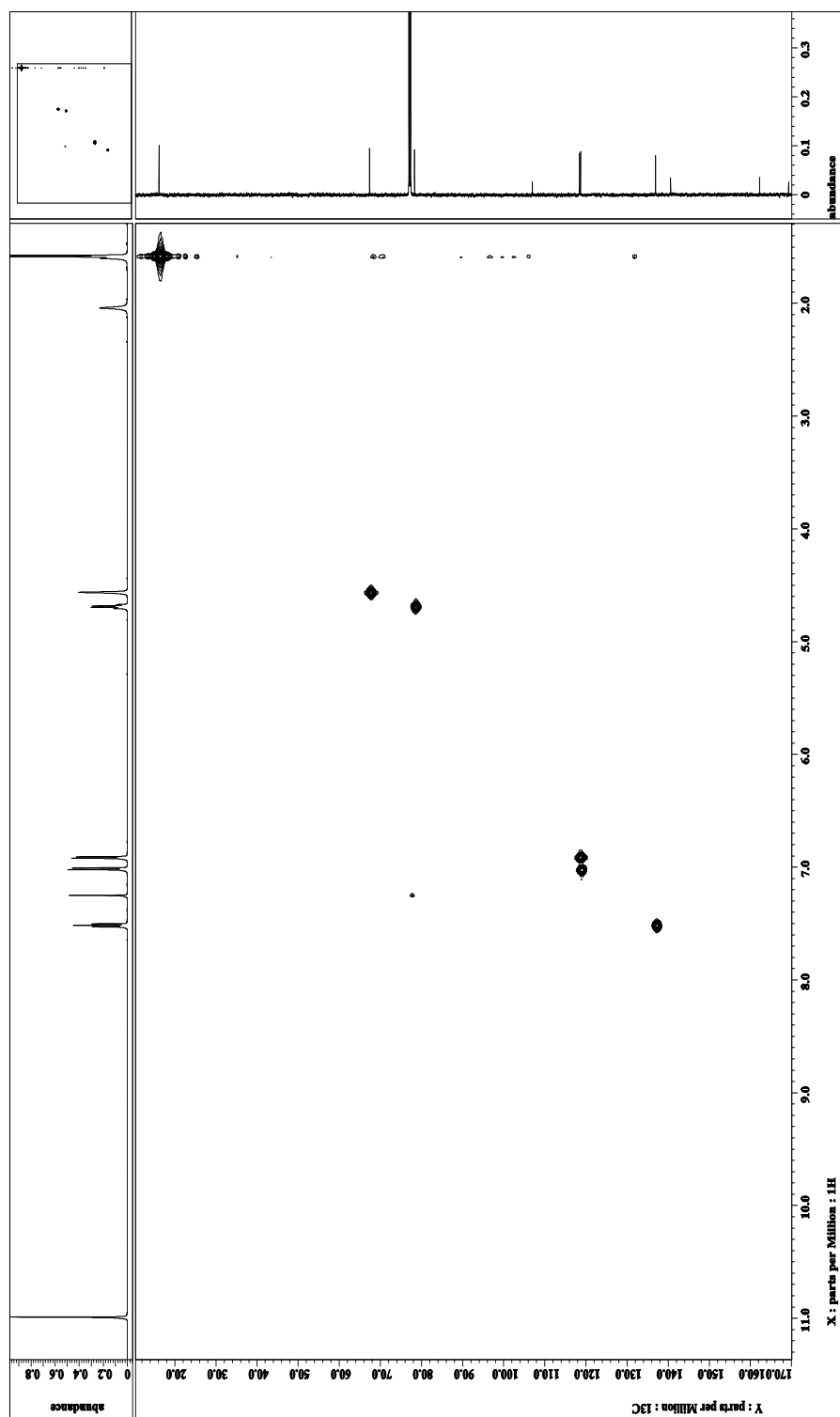


Figure 28 ^1H - ^1H COSY spectrum of *cis*-4-hydroxymellein

Figure 29 HMQC spectrum of *cis*-4-hydroxymellein

Chapter 3 Secondary metabolites from fungus X.B315

3.1 *Xylaria* species B315 Profile

The fungus coded **X.B315** was collected in Thailand and classified as *Xylaria* species. It was received in Petri dishes as shown in **Figure 30**. The front view of the Petri dish showed a white mycelium. The reverse side of the fungus was orange with black circular spots.

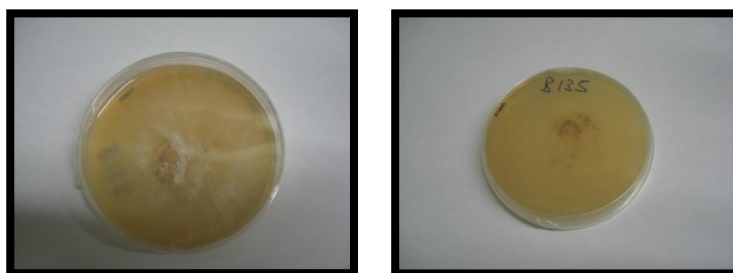
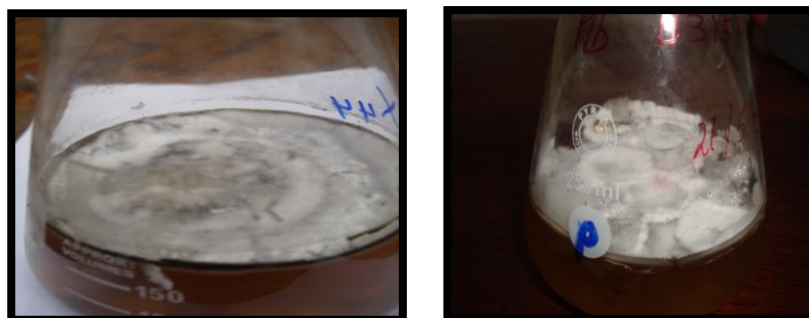


Figure 30 Fungus X.B315 as received from Thailand

A satisfactory production of secondary metabolites depend on the medium the fungus is cultured on; the more complex a medium is, the more chemical diversity the fungi will display¹⁷. Fungus **X.B315** was static sub-cultured at the same time on two different media; malt extract-glucose mixture and potato-yeast extract-glucose mixture. The fungus was grown into 10 conical flasks (250 ml), five contained an aqueous solution of malt extract, whilst the others contained potato based medium. The conical flasks were kept under observation for 14 days. Fungus **X.B315** growing on malt-extract developed a white mycelium with a black underside, whilst the same fungus growing on the potato-yeast extract produced a white fluffy mycelium with a pale yellow gelatinous underside. No fruiting bodies formed on either media. **Figure 31** shows the growth patterns of the fungus on malt and potato extracts after 14 days.



Malt-extract-glucose

Potato-yeast extract-glucose

Figure 31 Fungus X.B315 in conical flask after 2 weeks

The fungi were then cultured in (15 x 1 L) Thompson bottles, each bottle containing about (500 ml) of the aqueous medium. The mature fungus, which grew on the malt extract, produced a white gelatine-like mycelium with black underside, whilst the fungus which was cultured on potato-yeast extract produced a cotton-like mycelium

Figure 32.

Malt-extract-glucose

Potato-yeast extract-glucose

Figure 32 The mycelia of fungus X.B315

The matured cultures were harvested and the mycelia recovered by filtration through a muslin cloth. The aqueous filtrates of both malt and potato extracts were extracted with ethyl acetate using a separating funnel. The solvent fractions were dried over anhydrous sodium sulphate. Evaporation of the solvent *in vacuo* yielded (5.2 g) of malt crude extract and (7.2 g) of potato crude extract.

The two crude extracts were screened using TLC for their metabolite profile. A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. Both extracts showed matching patterns of spots under visible and UV light. Under visible light, a violet coloured spot appeared at R_f 0.32. Under short wave UV (245 nm), two active spots were detected; at R_f 0.92 and 0.64. When the TLC plates were sprayed with diazotised *p*-nitroaniline the two spots at (R_f 0.92 and 0.64) developed a strong orange colouration, whilst the spot at R_f 0.32 showed a yellowish brown colouration. The spot at R_f 0.32 developed an intense yellow colouration with anisaldehyde spray reagent, which turned to red after heating 2-3 min at 110 °C. Comparison of the spot patterns for the metabolite profile for the fungus grown on the malt and potato extracts, suggested that both produced the same components.

3.2 Isolation of secondary metabolites from fungus X.B315 on malt extract

3.2.1 Isolation of Cytochalasin D

The ethyl acetate crude extract (5.2 g) was triturated with ethyl acetate and the mixture left overnight. A white precipitate formed which was filtered off and recrystallised from methanol to give colourless needles (65 mg), mp 238-240 °C, ES $[M+H]^+$ m/z 508. This compound showed an opaque spot, when treated with diazotised *p*-nitroaniline spray reagent at R_f 0.27 on TLC. The 1H NMR spectrum (C_5D_5N) showed a characteristic N-H signal at δ_H 9.01. The spectral and physical data of this compound confirmed this compound was cytochalasin D the prior identification of which is described in **Chapter 2**.

3.2.2 Isolation of *R*-Mellein

After removal of cytochalasin D, the ethyl acetate crude extract (3.7 g) was chromatographed on four PTLC plates (20 cm x 80 cm); (1 g) on each plate. Each plate was prepared by mixing Merck Kieselgel GPF254 silica gel (100 g) with distilled water (270 ml) in a beaker (1 L) and left for 45 min to settle before spreading on the clean glass PTLC plate. After applying the extract on the plate; it was developed in a large tank containing toluene, ethyl acetate and acetic acid (500:450:10). When elution was complete, the plate was removed and air-dried and a band (1.0 cm) at each end of the plate was sprayed with diazotised *p*-nitroaniline spray reagent to detect the bands.

A UV active band at R_f 0.92 developed a strong orange colouration, when sprayed with *p*-nitroaniline spray reagent suggesting a phenolic compound, but it was not active with anisaldehyde spray reagent. The band was recovered and washed with ethyl acetate to produce a yellow solution.

Evaporation of the solvent yielded a yellow oil (35 mg). Trituration of the oil with *n*-hexane gave a white solid (12 mg), which was recrystallised twice from the same solvent to yield colourless plates (3 mg), mp 52-53°C, ES $[M+H]^+$ m/z 179, $[\alpha]_D^{25}$ -100° (c 1, in EtOH). The 1H NMR spectrum ($CDCl_3$, Figure 34) shows ten proton resonances δ_H 1.52 (3H, d, J 6.3 Hz), 2.93 (2H, d, J 7.0 Hz), 4.69-4.75 (1H, m), 6.67 (1H, d, J 7.3 Hz), 6.87 (1H, d, J 8.4 Hz), 7.39 (1H, t, J 7.5 Hz) and 11.02 (1H, s, OH). The 3 sets of signals in the aromatic region indicated a 1,2,3- tri-substituted ring. The signal at δ_H 11.02 suggests a chelated hydroxyl group. The signals at δ_H 1.52, 4.69 and 2.93 represent a CH_3CHCH_2 subunit.

In the ^{13}C NMR spectrum ($CDCl_3$, Figure 35) ten carbon signals comprise a methyl at δ_c 20.88, a methylene at δ_c 34.73, four methine carbons at δ_c 76.21, 116.36, 118.00

and 136.25 and four quaternaries at 108.41, 139.50, 162.31 and 170.07. The mass spectrum, ^1H and ^{13}C NMR data, suggested a molecular formula of $\text{C}_{10}\text{H}_{10}\text{O}_3$. Comparison of the spectral data, mp and the optical rotation of this compound with reported data identified this compound as *R*-mellein^{33, 39, 88, 95} **Figure 33**.

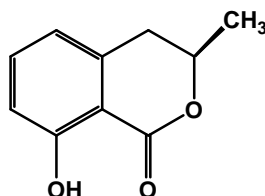


Figure 33 *R*-mellein structure

^1H , ^{13}C and DEPT-135 NMR data of mellein is given in **Table 9**. The structure elucidation of mellein is described in more detail in **Chapter 2**.

Table 9 ^1H and ^{13}C NMR data of *R*-mellein (CDCl_3)

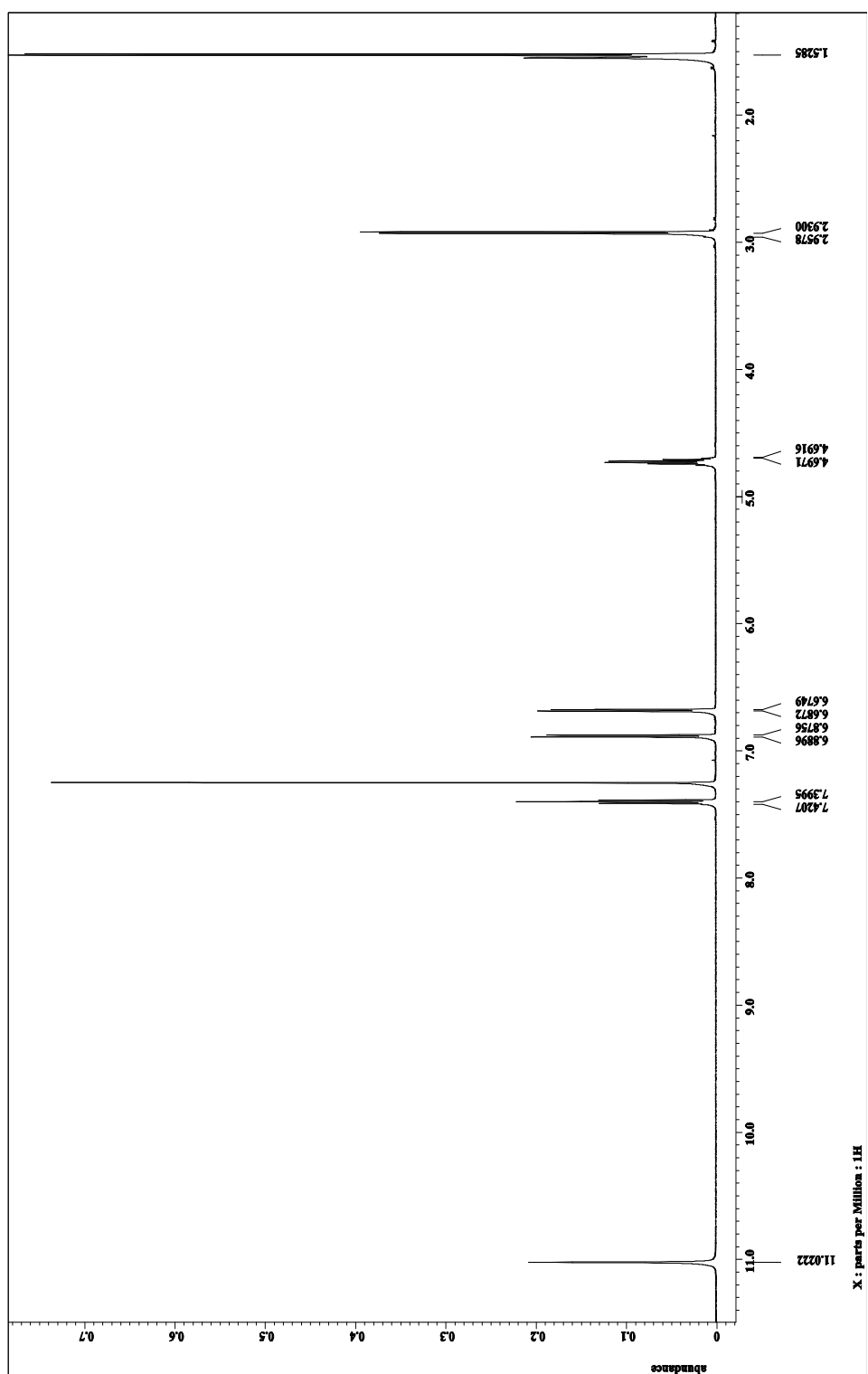
N_O	δ_C	δ_H
1	170.07-C	
3	76.21-CH	4.69-4.75 (1H, m)
4	34.73-CH ₂	2.93 (2H, d, <i>J</i> 7.0 Hz)
5	118.00-CH	6.87 (1H, d, <i>J</i> 8.4 Hz)
6	136.25-CH	7.39 (1H, t, <i>J</i> 7.5 Hz)
7	116.36-CH	6.67 (1H, d, <i>J</i> 7.3 Hz)
8	162.31-C	
9	108.41-C	
10	139.50-C	
11	20.88-CH ₃	1.52 (3H, d, <i>J</i> 6.3 Hz)
*	OH group	11.02 (1H, s)

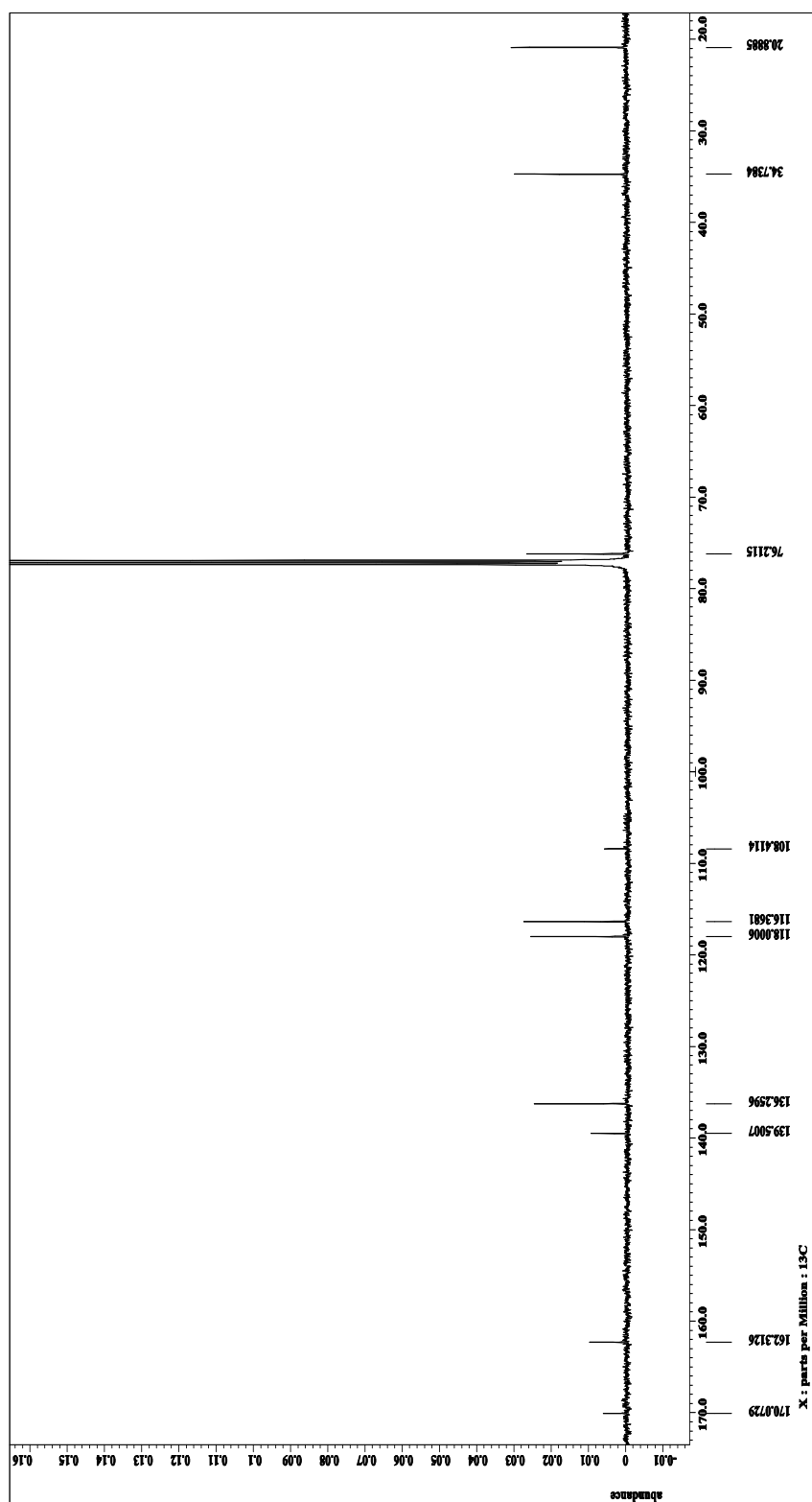
Mellein of unidentified stereochemistry was first isolated in 1933 from *Aspergillus melleus*^{39, 88}. Later researchers worked out the stereochemistry of mellein. It was found to exist naturally in two main forms; *R*-(-)-mellein and *S*-(+)-mellein. It has been isolated at Bradford from different *Xylaria* fungi such as *X. longiana*³³, *X. grammica*³⁵ and *X. badia*³⁷. In this project *R*-(-)-mellein has been found to be produced by the *Xylaria* fungus **B315**.

In **Table 10**, a summary is presented showing the source and physical properties of naturally occurring *R*-(-)-mellein, which have been reported in the literature.

Table 10 Source and physical properties of *R*-(-)-mellein reported in literature

Melting point	Optical rotation	Source
56 °C	[α] -102° (<i>c</i> 1, CHCl ₃)	<i>Aspergillus onika</i> ⁹⁵
58 °C	[α] -100° (<i>c</i> 1, CHCl ₃)	<i>Hypoxyton howieanum</i> ⁸⁹
56-58 °C	[α] -80° (<i>c</i> 0.02, CHCl ₃)	<i>Aspergillus melleus</i> ³⁹
47-48 °C	[α] -94° (<i>c</i> 0.48, CHCl ₃)	<i>Lasiodiplodia spp.</i> ⁴¹
57 °C	[α] -96° (<i>c</i> 1, CHCl ₃)	<i>Xylaria longiana</i> ³³

Figure 34 ^1H NMR spectrum of mellein

Figure 35 ^{13}C NMR spectrum of mellein

3.2.3 Isolation of 4-Hydroxymellein as a stereoisomeric mixture

The next UV active band on the PTLC plate, which had an R_f of 0.64, developed a strong orange colouration with the *p*-nitroaniline spray reagent; suggesting a phenolic compound. It was isolated as a pale yellow oil and recrystallised from petroleum ether (bp 80-100 °C) as fine colourless needles (4 mg), mp 121-125 °C, ES $[M+H]^+$ m/z 195.

The ^1H and ^{13}C NMR spectra (CDCl_3 , Figure 37& 38) are characteristic of a mellein-type molecule, which exists as a mixture of two isomeric forms in unequal ratio (3:1). The C-4 methylene signals of mellein are absent and have been replaced by an oxygenated methine signal at δ_{H} 4.55/4.56 and δ_{C} 67.29/69.19. Replacement of the methylene group with a hydroxyl-methine group at position 4 in mellein molecule gives 4-hydroxymellein ($\text{C}_{10}\text{H}_{10}\text{O}_4$). Comparison of the spectral data for this compound with reported data^{93, 101} for 4-hydroxymellein confirms this compound as 4-hydroxymellein **Figure 36**. NMR data of 4-hydroxymellein are listed in **Table 11**.

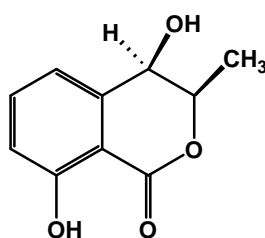


Figure 36 4-Hydroxymellein structure

Table 11 NMR data for 4-Hydroxymellein (CDCl₃)

Experimental results				From <i>X. longiana</i> ¹⁰¹	
N _O	δ _C	DEPT	δ _H	δ _C	δ _H
1	169.29	C		169.24	
1	168.59	C		169.32	
3	78.29	CH	4.60 m	78.30	4.61 qd
3	80.05	CH	4.57 m	80.02	4.70 q
4	67.29	CH	4.56 m	67.19	4.61 d
4	69.19	CH	4.55 m	69.06	4.70 d
4-OH			2.14 (OH, s)		
5	118.39	CH	6.91 m	118.38	6.95 d
5	118.60	CH	6.90 m	118.48	7.01 dd
6	136.89	CH	7.01 m	136.83	7.53 qd
6	136.97	CH	7.00 m	136.90	7.55 qd
7	116.35	CH	7.52 m	116.36	7.01 dd
7	117.87	CH	7.49 m	117.75	6.95 d
8	162.04	C		161.94	
8	162.14	C		162.02	
9	106.73	C		106.66	
9	106.90	C		106.85	
10	140.58	C		140.54	
10	141.27	C		141.27	
11	16.09	CH ₃	1.56 (d)	16.02	1.52 (d)
11	18.00	CH ₃	1.49 (d)	17.94	1.60 (d)
*	O-H		10.98 (s)		10.99 (s)
*			10.95 (s)		11.01 (s)

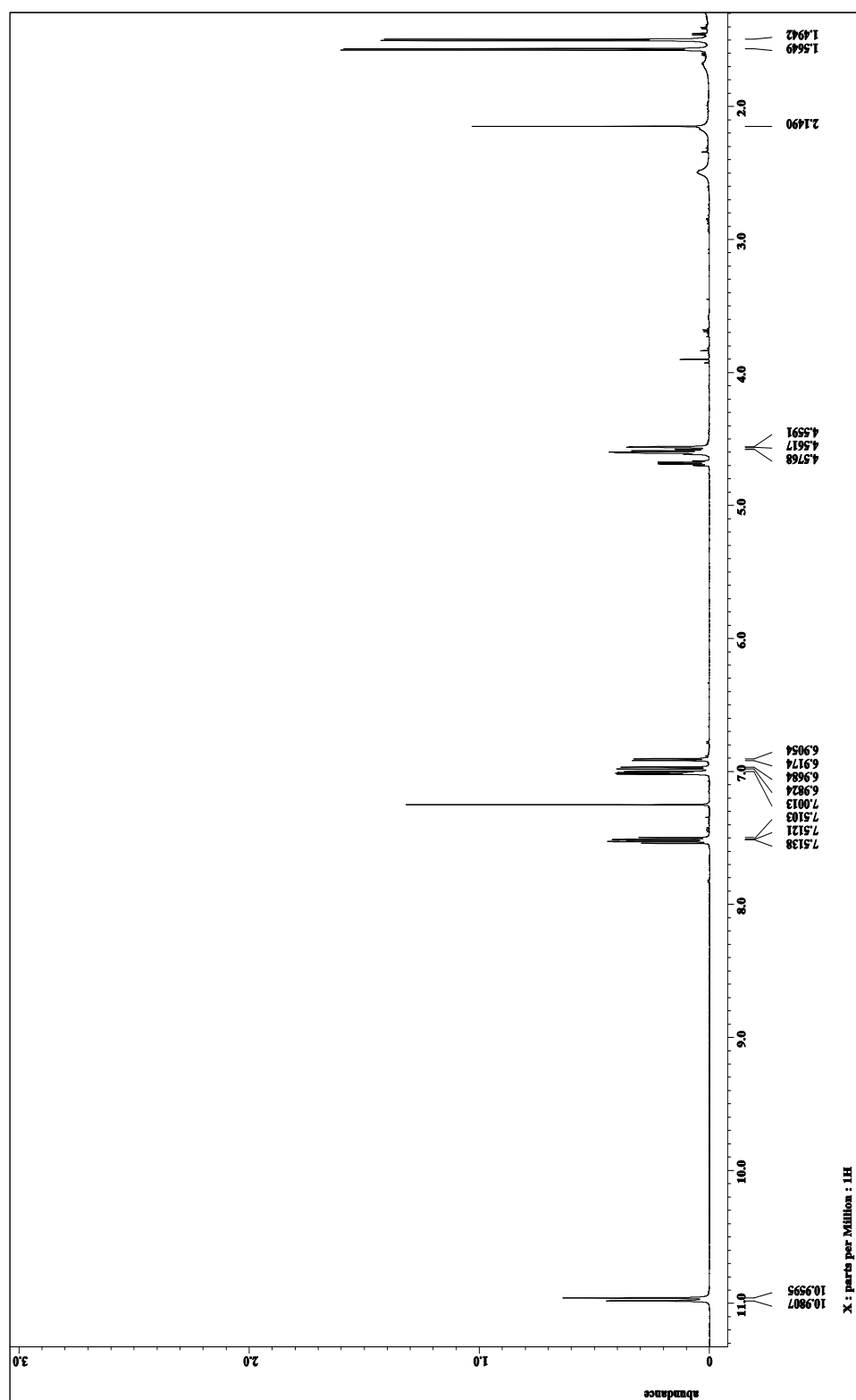
The ^1H NMR spectrum (CDCl_3 , Figure 37) of the isomeric mixture of *cis*- and *trans*-4-hydroxymellein showed six down-field signals at δ_{H} 7.52, 7.49, 7.01, 7.00, 6.91 and 6.90 and six up-field signals at δ_{H} 4.60, 4.57, 4.56, 4.55, 1.56 and 1.49 as well as two highly deshielded singlets at δ_{H} 10.98 and 10.95.

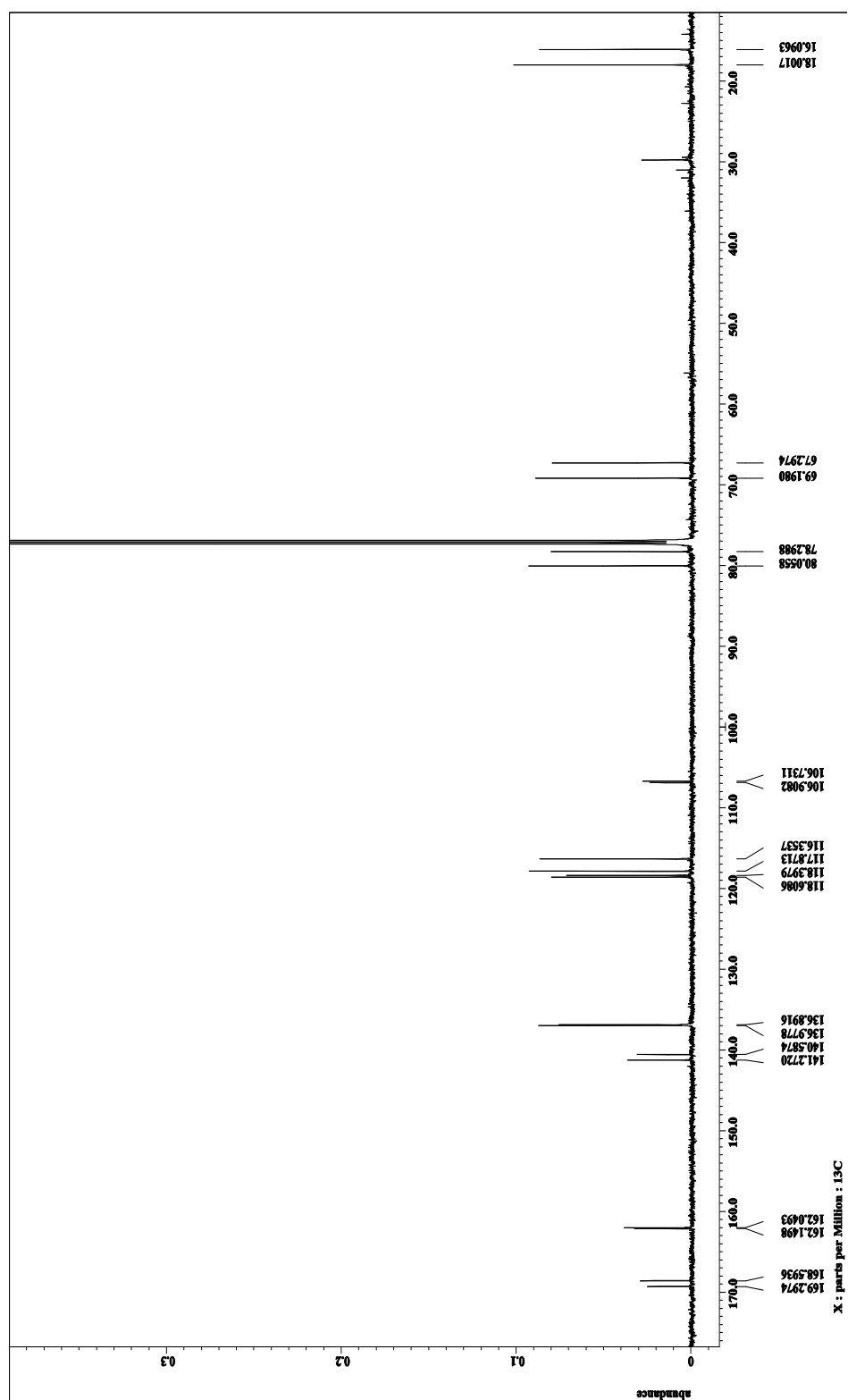
The *cis*-configuration was assigned to H-3 and H-4 on the basis of the small coupling constant (2.04 Hz), whereas the analogous *trans*-isomer had a higher value (4.5 Hz).

Septoria nodorum^{97, 98}, a phytopathogenic fungus, produces the diastereoisomers *cis* and *trans* 4-hydroxymelleins. The isolation of *trans*-(3*R*,4*S*)-4-hydroxymellein from *Moringa oleifera*⁹⁸ was the first report of this metabolite from the stems of a plant species. *Cercospora taiwanensis*⁹⁶, a well known crop pathogen, produces *cis*-(3*S*,4*S*)-4-hydroxymellein in addition to (+)-mellein. The *cis*-isomer has been isolated previously in Bradford from *X. longiana*¹⁰¹, *X. grammica*³⁵ and other *Xylaria* species⁸⁷. In this project, the *cis*-3*R*,4*R*-4-hydroxymellein was isolated from endophytic fungi described in **Chapter 2**. Different 4-hydroxymellein stereoisomers which have been isolated from several fungi are illustrated in **Table 12**.

Table 12 Different 4-hydroxymellein stereoisomers

Isomer	mp/ °C	[α]	Source
(3 <i>R</i> ,4 <i>R</i>)(-)- <i>cis</i> -form	123-124	-31	<i>Botryospaeria obtuse</i> ¹⁰² <i>Septoria nodorum</i> ¹⁰³
(3 <i>S</i> ,4 <i>S</i>)(+)- <i>cis</i> -form	131-132	+37	<i>Cercospora taiwanensis</i> ⁹⁶
(3 <i>R</i> ,4 <i>S</i>)(-)- <i>trans</i> -form	131-132	-29	<i>Moringa oleifera</i> ⁹⁸

Figure 37 ¹H NMR spectrum of 4-hydroxymellein

Figure 38 ^{13}C NMR spectrum of 4-hydroxymellein

3.2.4 Isolation of Phloroglucinol

A compound, which gave a violet spot under visible light on the PTLC plate was at R_f 0.32, developed an intense yellow colouration with acetic acid, anisaldehyde and sulphuric acid (98:1:1) spray reagent, which turned to red after heating 2-3 min at 110 °C. It was isolated as a brown powder (420 mg) and recrystallised twice from water to give colourless crystals (350 mg), mp 214-216 °C, ES $[M+H]^+$ m/z 127, IR_{ATR} ν_{\max} cm⁻¹ 3461, 3160, 1609. The IR absorptions of this compound indicated the presence of hydroxyl groups and aromatic C=C band at 1609 cm⁻¹.

In the ¹H NMR spectrum (acetone-d₆, Figure 41) showed only two resonances at δ_H 5.84 (3H, s) and three hydroxyl protons as a singlet at δ_H 8.08 (OH, s). In the ¹³C NMR spectrum (C₅D₅N, Figure 42) two signals comprise three chemically and magnetically equivalent methine carbons at δ_C 94.55 and three equivalent quaternary carbons at δ_C 159.33. From the mass spectrum, ¹H and ¹³C NMR, the molecular formula was deduced to be C₆H₆O₃. The physical and spectral data of this compound was compared with the literature data¹⁰⁴ and identified this compound as 1,3,5-benzenetriol or phloroglucinol **Figure 40**.

ACD/LABS NMR prediction software (v.10) was used to assist the identification of the compound. The ¹H and ¹³C predictors were used to predict the spectra of the suggested structure of the compound. The calculated values of the chemical shifts were compared and showed good agreement with the experimental values **Figure 39**.

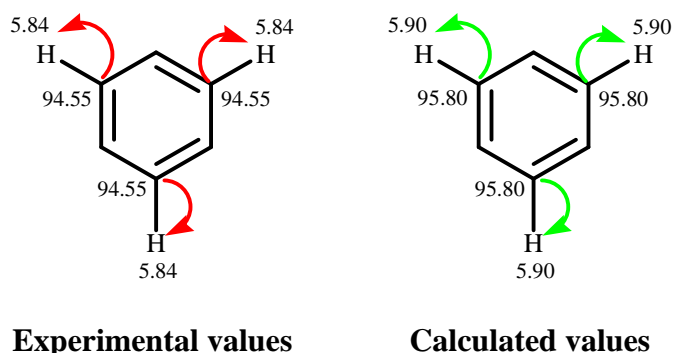


Figure 39 HMQC correlations for phloroglucinol

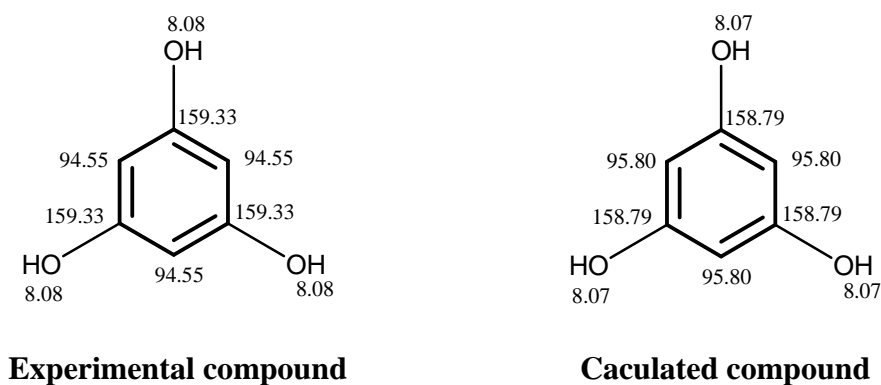


Figure 40 NMR data for phloroglucinol structure

Trihydroxybenzenes are phenolic compounds that are widely distributed in plants. They are isolated by extraction but can also be prepared by synthesis. These compounds are of great interest in medicinal chemistry because they exhibit, antiallergy¹⁰⁵, antitumor¹⁰⁶ and antiviral activity¹⁰⁷.

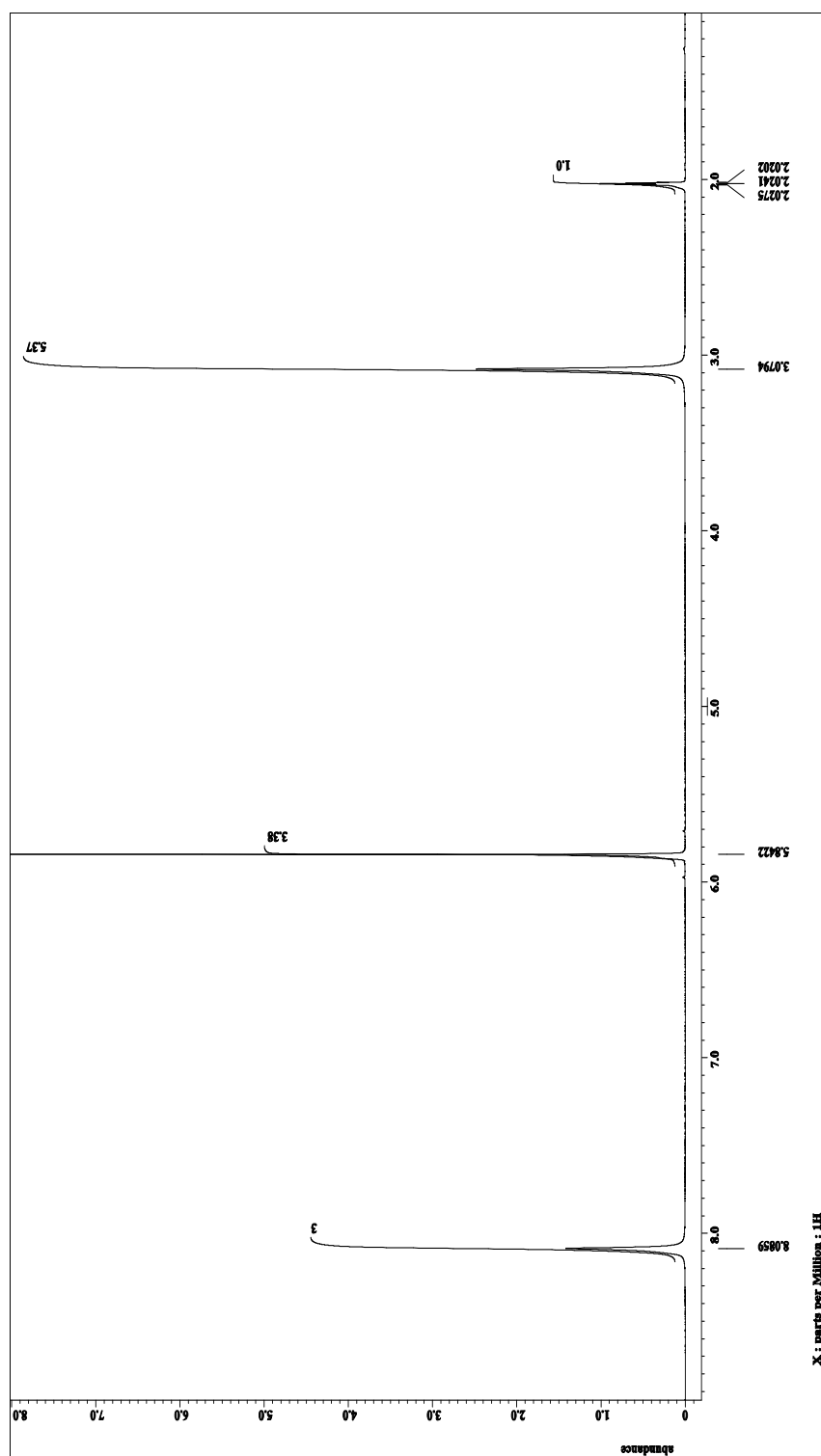
A *Eucalyptus* species, traditionally used as an Indian medicinal plant, were tested for its antimicrobial activity against certain drug-resistance bacteria and a yeast *Candida albicans*¹⁰⁸. An acylated form of phloroglucinol was isolated from *Helichrysum caespititium*¹⁰⁹. This compound inhibits the growth of the Gram-positive bacteria such as *Bacillus cereus* and *Staphylococcus aureus* and also inhibits the growth of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*¹⁰⁹.

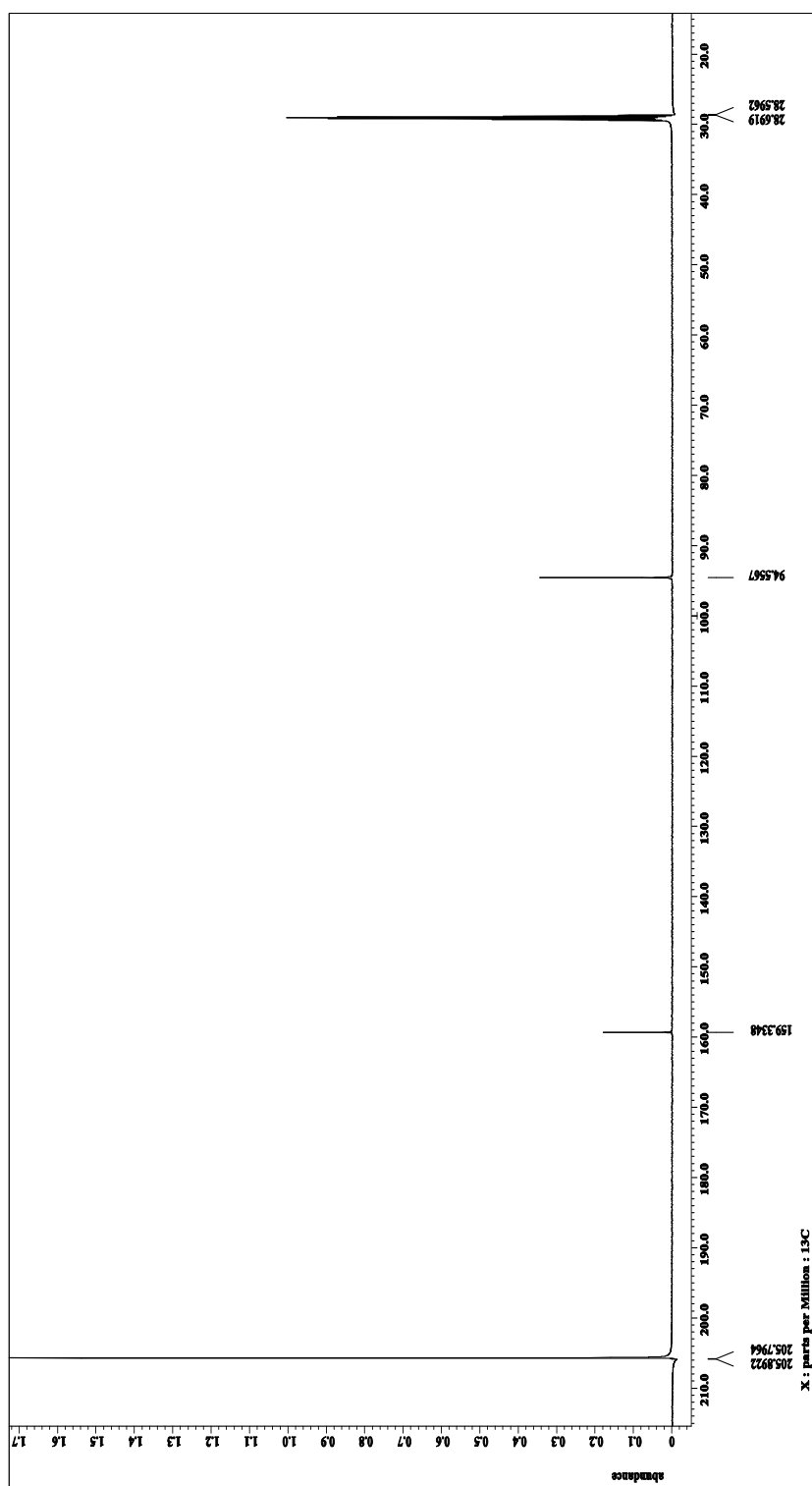
Phloroglucinol or 1,3,5-benzenetriol derivatives, which have been isolated from the aerial parts of *Hypericum calycinum*¹¹⁰ shows fungicidal activity against *Cladosporium cucumerinum*, and antimalarial activity in an *in vitro* test system. Also, derivatives of phloroglucinol have been isolated and identified from the marine algae genus *Zonaria*¹¹¹. This appears to be the first report of the isolation of phloroglucinol from a *Xylaria* species.

3.3 Isolation of Phloroglucinol from X.B315 mycelium

The air-dried mycelium was pulverised (65.5 g). The sample was packed into a soxhlet thimble, which was placed into a soxhlet apparatus. It was extracted with chloroform (400 ml) for 16 h. After the evaporation of the solvent *in vacuo* a brown crude extract (5.6 g) was obtained.

The extract was then analysed for its content using TLC. A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. A visible violet spot was detected on a TLC plate at R_f 0.32. The spot developed a yellowish brown colouration with *p*-nitroaniline spray reagent, whilst it showed a yellow colouration with anisaldehyde spray reagent, which turned to red after heating 2-3 min at 110 °C. The crude extract was warmed with H₂O (15 ml) for 10 min. The solution was filtered off and filtrate was left to cool. Colourless crystals of phloroglucinol formed (450 mg). It appears that phloroglucinol is a major secondary metabolite produced by fungus **X.B315**.

Figure 41 ^1H NMR spectrum of phloroglucinol

Figure 42 ^{13}C NMR spectrum of phloroglucinol

3.4 Secondary metabolites from fungus *X.B315* on potato-extract

Analogous procedures to those described above for the isolation of secondary metabolites of *X.B315* grown on malt extract-glucose were applied to the ethyl acetate crude extract of the same fungus grown on potato medium. It was found that fungus *X.B315* grown on potato-yeast extract-glucose mixture produced the same secondary metabolites, but in higher yield. The mycelium of this fungus also produced phloroglucinol. The yields of the metabolites produced by fungus *X.B315* on different media are summarised in **Table 13**.

Table 13 Yields of metabolites produced by fungus *X.B315*

Metabolites	Malt-extract	Potato-extract
Cytochalasin D	65 mg	113 mg
<i>R</i> -Mellein	3 mg	12 mg
4-Hydroxymellein stereo-isomeric mixture	4 mg	7 mg
Phloroglucinol	350 mg	419 mg
Phloroglucinol from <i>X.B315</i> mycelium	450 mg	570 mg

Chapter 4 Secondary metabolites from fungus X.62

4.1 *Xylaria* endophyte X.62 Profile

The fungus coded **X.62** was isolated from palm tree leaves in Thailand and classified as a *Xylaria* endophyte. It was received in Petri dishes as shown in **Figure 43**. The front view of the Petri dish showed a white mycelium. The reverse side of the fungus was also white; but with a black ring round the edge of the dish.

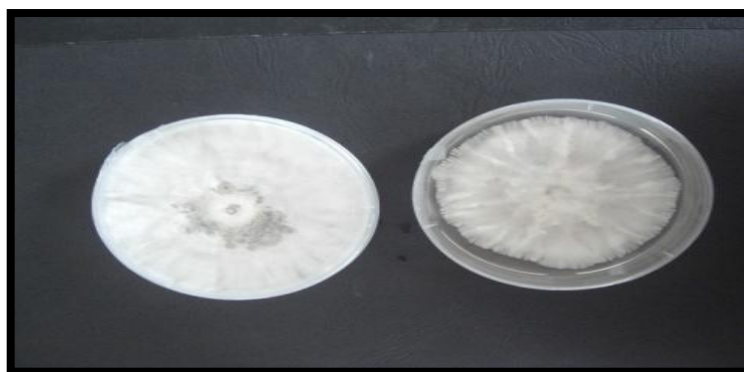


Figure 43 Fungus X.62 as received from Thailand

The fungus was static sub-cultured into four conical flasks (250 ml). It produced a white mycelium after 2 weeks. The fungus was then cultured on a malt-yeast extract glucose mixture into Thompson bottles (15 x 1L) for 8 weeks. During the first 4 weeks it grew as a white mycelium with black areas underside.

The matured cultures were harvested and the mycelia were recovered by filtration through a muslin cloth and left to air-dry. The aqueous filtrate was extracted with ethyl acetate in a separating funnel (5 L). The ethyl acetate fraction was dried over anhydrous sodium sulphate. After the removal of the solvent, a brown crude extract was obtained (1.2 g).

TLC analysis of the crude extract was conducted in different solvent systems. The resultant plates showed that the solvent systems; toluene, ethyl acetate and acetic acid (50:49:1), chloroform and methanol (95:5) and petroleum ether (bp 60-80°C), ethyl acetate and acetic acid (50:49:1) showed that the crude extract contained a

complex mixture of different components with different R_f values. When the plates were sprayed with *p*-nitroaniline spray reagent opaque spots were revealed. ^1H NMR spectroscopy ($\text{C}_5\text{D}_5\text{N}$) showed that the crude extract contained a mixture of cytochalasins according to four N-H signals. Several attempts to isolate these compounds by chromatographic techniques failed.

4.2 Isolation of secondary metabolites from fungus X.62 mycelium

4.2.1 Isolation of 19,20-Epoxyoctachalasin C

The air-dried black mycelium (79.3 g) was pulverised. The powdered mycelium was packed firmly into a soxhlet thimble, which was placed into a soxhlet apparatus. The mycelium sample was extracted with chloroform (400 ml) for 16 h. After the evaporation of the solvent *in vacuo*, a brown gummy solid was obtained (5.0 g). The extract was then analysed for its content by TLC.

A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. Visualization under short wave UV (245 nm) showed a single spot with R_f 0.42. When the TLC plate was sprayed with *p*-nitroaniline reagent an opaque spot was observed at R_f 0.42, which indicated that the compound was likely to be a cytochalasin. The ethyl acetate extract was triturated with ethyl acetate and left overnight. A white solid was obtained. The solid was filtered off and recrystallised from methanol to yield white needles, mp 266-270 °C, ES $[\text{M}+\text{H}]^+$ m/z 524, $[\alpha]_D^{25}$ -6.4° (c 1, in CHCl_3). The IR_{ATR} spectrum of this compound showed absorptions at: 3423, 1744 and 1697 cm^{-1} , which suggested the presence of the following groups, a hydroxyl, a carbonyl ester and an amide.

The ^1H NMR spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 49) of this compound gave the general appearance of that expected for a cytochalasin. A molar mass of 523 further supported this conclusion. The spectrum showed five methyl signals; one doublet at

δ_{H} 0.98 and four singlets at δ_{H} 1.24, 1.59, 1.83, 2.10 and a N-H singlet signal at δ_{H} 9.46, which is characteristic for a cytochalasin structure. The spectrum is different to that of cytochalasin D, which was discussed in **Chapter 2**. The absence of the alkene protons at δ_{H} 5.04 and 5.40 (12-Ha & 12-Hb) and the associated carbon signal at δ_{C} 112.16 (CH_2 , C-12) confirmed that this compound is not cytochalasin D.

The presence of five methyl groups in a pattern of a doublet and four singlets suggested that this compound could be related to cytochalasin C **Figure 44**.

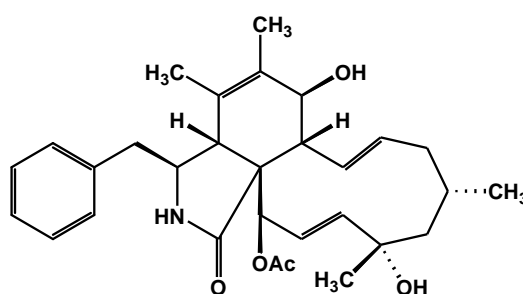


Figure 44 Cytochalasin C

The unknown compound has a molar mass of 523, whilst cytochalasin C has a molar mass 507. The difference of 16 mass units strongly pointed to the presence of an additional oxygen.

The ^{13}C NMR spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 50) of this compound presents 28 carbon signals. The signals at δ_{C} 128.84 and 129.88 are double the height of other peaks signifying that these signals are each due to two carbon atoms to give a total of 30 carbons as found in cytochalasin C. Comparison of the ^{13}C NMR spectral data for cytochalasin C²⁵ and this unknown **Table 16** revealed that the carbon signals associated with 19,20 $\text{C}=\text{C}$ were absent and that there were two additional methine signal at δ_{C} 54.11 and 61.45 indicative of sp^3 carbons attach to oxygen. This information together with the presence of additional oxygen suggested that the 19,20

double bond was now epoxidized, indicative of the unknown being 19,20-epoxycytochalasin C **Figure 45**.

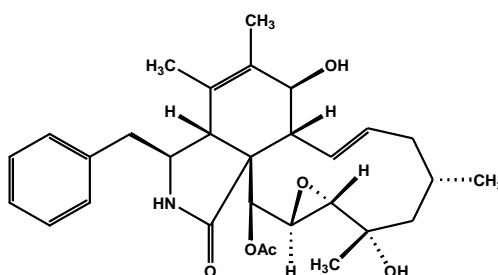


Figure 45 19,20-Epoxycytochalasin C

^1H and ^{13}C NMR data of this cytochalasin are presented in **Table 15**.

It is not possible from the spectral data for the double bond to be at position C-13 and C-14. The chemical shift of the N-H proton of the lactam ring in cytochalasin compounds appears generally above δ_{H} 9.0, when pyridine- d_5 is used as the NMR solvent. It has been observed that the signal for this proton can be characteristic for cytochalasin. In 19,20-epoxycytochalasin C for instance, the N-H proton resonates at δ_{H} 9.46, which is the case for the isolated cytochalasin.

Table 14 shows the characteristic physical data of some epoxycytochalasins.

Table 14 Characteristic physical data of some epoxycytochalasins^{25,110}

Epoxycytochalasins	$[\alpha]$	m/z
19,20-epoxycytochalasin R	-60°	524
19,20-epoxycytochalasin N	-55°	540
19,20-epoxycytochalasin C	-300°	524
19,20-epoxycytochalasin D	-228°	524

Table 15 NMR data of 19,20-epoxycytochalasin C (C₅D₅N)

N _O	δ _H	δ _C	DEPT
1		175.35	C
2	9.46 (1H, s)	NH	
3	3.59 (1H, t, <i>J</i> 7.2 Hz)	50.08	CH
4	3.17 (1H, d, <i>J</i> 7.3 Hz)	51.03	CH
5		126.58	C
6		133.72	C
7	4.40 (1H, s)	68.89	CH
7-OH	4.97 (1H, s)		
8	2.16 (1H, s)	52.82	CH
9		60.75	C
10	2.82-2.84 (2H, m)	45.28	CH ₂
11	1.83 (3H, s)	14.78	CH ₃
12	1.24 (3H, s)	17.06	CH ₃
13	5.85-5.89 (1H, m)	132.21	CH
14	6.73-6.77 (1H, m)	132.89	CH
15a	1.94 (1H, dd, <i>J</i> 11.1, 12.2 Hz)	38.35	CH ₂
15b	2.60-2.63 (1H, m)		
16	2.85-2.88 (1H, m)	42.00	CH
17		216.19	C
18		77.16	C
19	3.75 (1H, s)	54.11	CH
20	4.20 (1H, s)	61.45	CH
21	6.26 (1H, s)	73.36	CH
22	0.98 (3H, d, <i>J</i> 6.5 Hz)	19.05	CH ₃
23	1.59 (3H, s)	20.38	CH ₃
24		170.84	C
25	2.10 (3H, s)	22.55	CH ₃
1'		138.74	C
2',6'	7.20-7.35 (2H, m)	129.88	CH
3',5'	7.20-7.35 (2H, m)	128.84	CH
4'	7.20-7.35 (1H, m)	126.81	CH

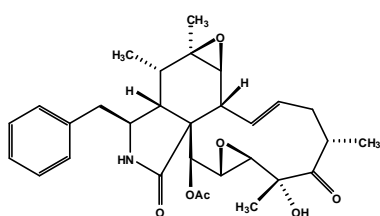
Table 16 ^{13}C NMR of 19,20-epoxycytochalasin C and cytochalasin C

N_O	19,20-Epoxycytochalasin C	Cytochalasin C²⁵
1	175.35	175.71
2	NH	NH
3	50.08	50.71
4	51.03	50.71
5	126.58	126.69
6	133.72	134.06
7	68.89	69.13
8	52.82	53.76
9	60.75	61.05
10	45.28	42.54
11	14.78	14.92
12	17.06	17.29
13	132.21	130.00
14	132.89	132.93
15	38.35	38.72
16	42.00	42.54
17	216.19	210.94
18	77.16	78.62
19	54.11	132.78
20	61.45	133.54
21	73.36	76.39
22	19.05	19.49
23	20.38	20.71
24	170.84	170.93
25	22.55	24.77
1'	138.74	138.99
2',6'	129.88	129.07
3',5'	128.84	128.60
4'	126.81	127.04

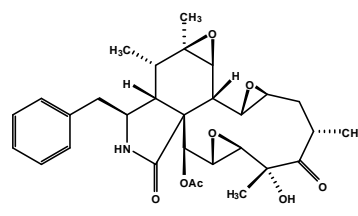
Cytochalasins are fungal metabolites which have been isolated from different genera of fungi such as *Phomopsis*, *Chaetomium*, *Hypoxylon* and most recently from *Xylaria* and *Daldinia*³⁰. Cytochalasins exhibit several biological activities including marked cytostatic effects on mammalian cells in tissue cultures^{112,113}, antibiotic and antitumor activity¹¹⁴.

19,20-Epoxychocthalasin C is a natural product. It was isolated and identified from the fungus *Xylaria hypoxylon*³⁰ alongside 19,20-epoxychocthalasin Q, 19,20-epoxychocthalasin R, 19,20-epoxychocthalasin D and 19,20-epoxychocthalasin N

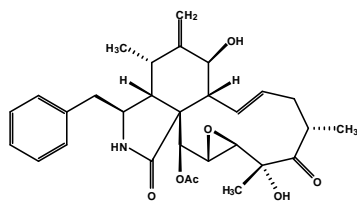
Figure 46.



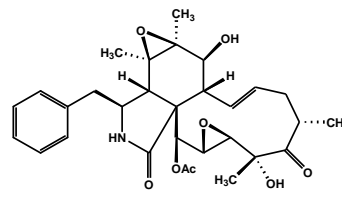
19,20-Epoxychocthalasin Q



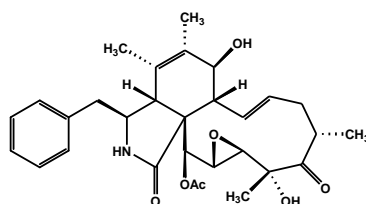
19,20-Epoxychocthalasin R



19,20-Epoxychocthalasin D



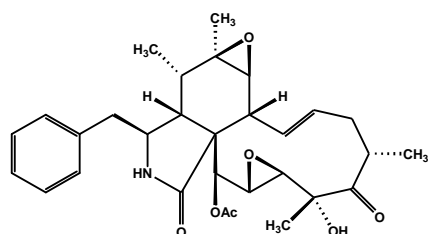
19,20-Epoxychocthalasin N



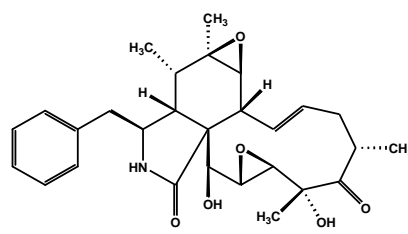
19,20-Epoxychocthalasin C

Figure 46 Epoxychocthalasins from *X. hypoxylon*

Dagne¹¹⁵ *et al* reported the isolation and characterisation of two new epoxycytochalasins; 19,20-epoxycytochalasin Q and deacetyl-19,20-epoxycytochalasin Q from *Xylaria obovata*, a tropical fungus was collected from the Munsea forest in Ethiopia **Figure 47**.



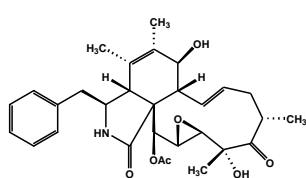
19,20-Epoxycytochalasin Q



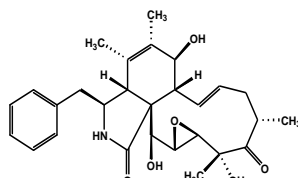
Deacetyl-19,20-epoxycytochalasin Q

Figure 47 Epoxycytochalasins from *X. obovata*

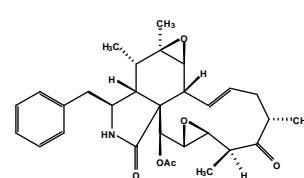
Another group of workers, Abate¹¹⁶ *et al* isolated 19,20-epoxycytochalasin C, deacetyl-19,20-epoxycytochalasin C and xylobovatin from cultures of the same fungus **Figure 48**.



19,20-Epoxycytochalasin C



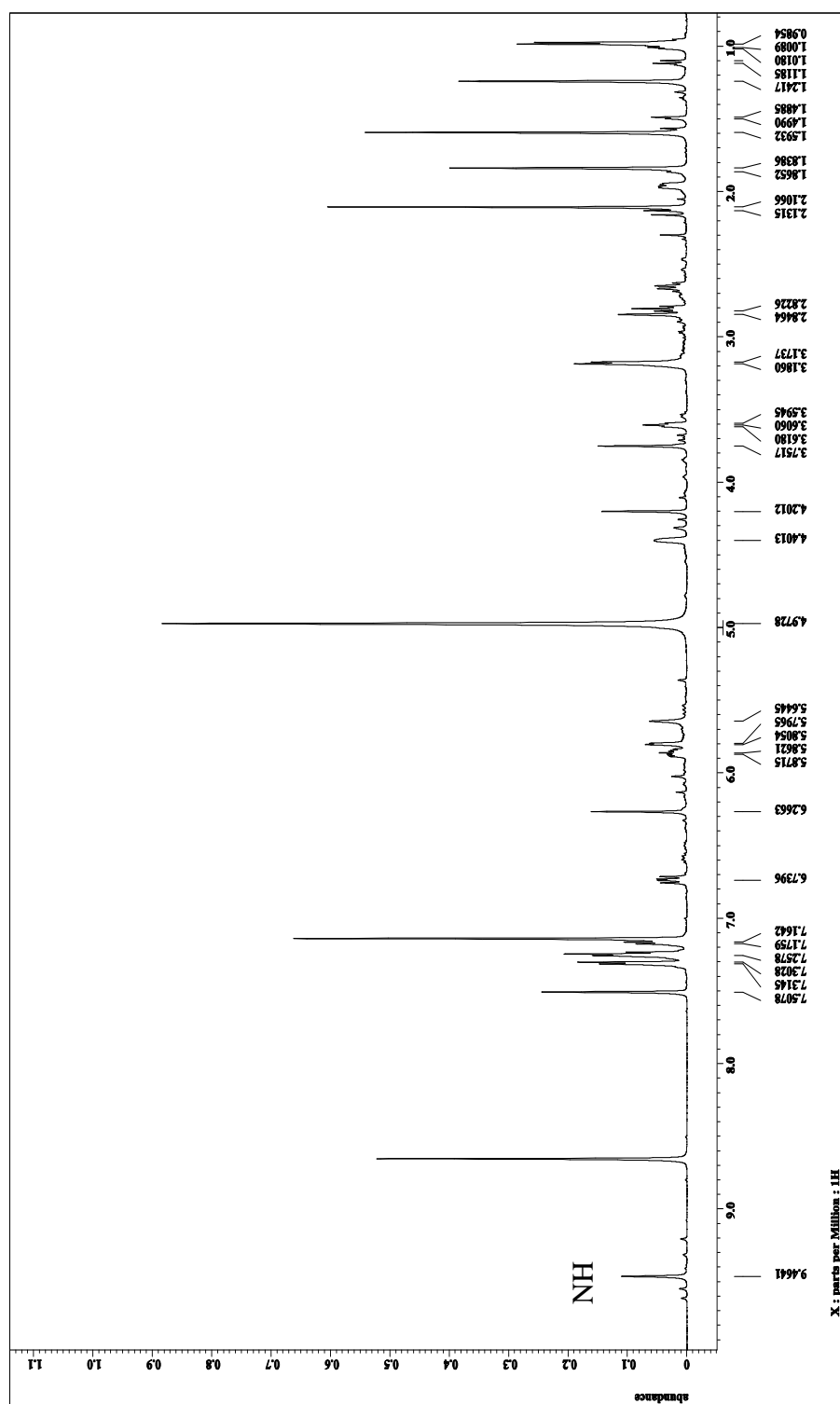
Deacetyl-19,20-epoxycytochalasin C

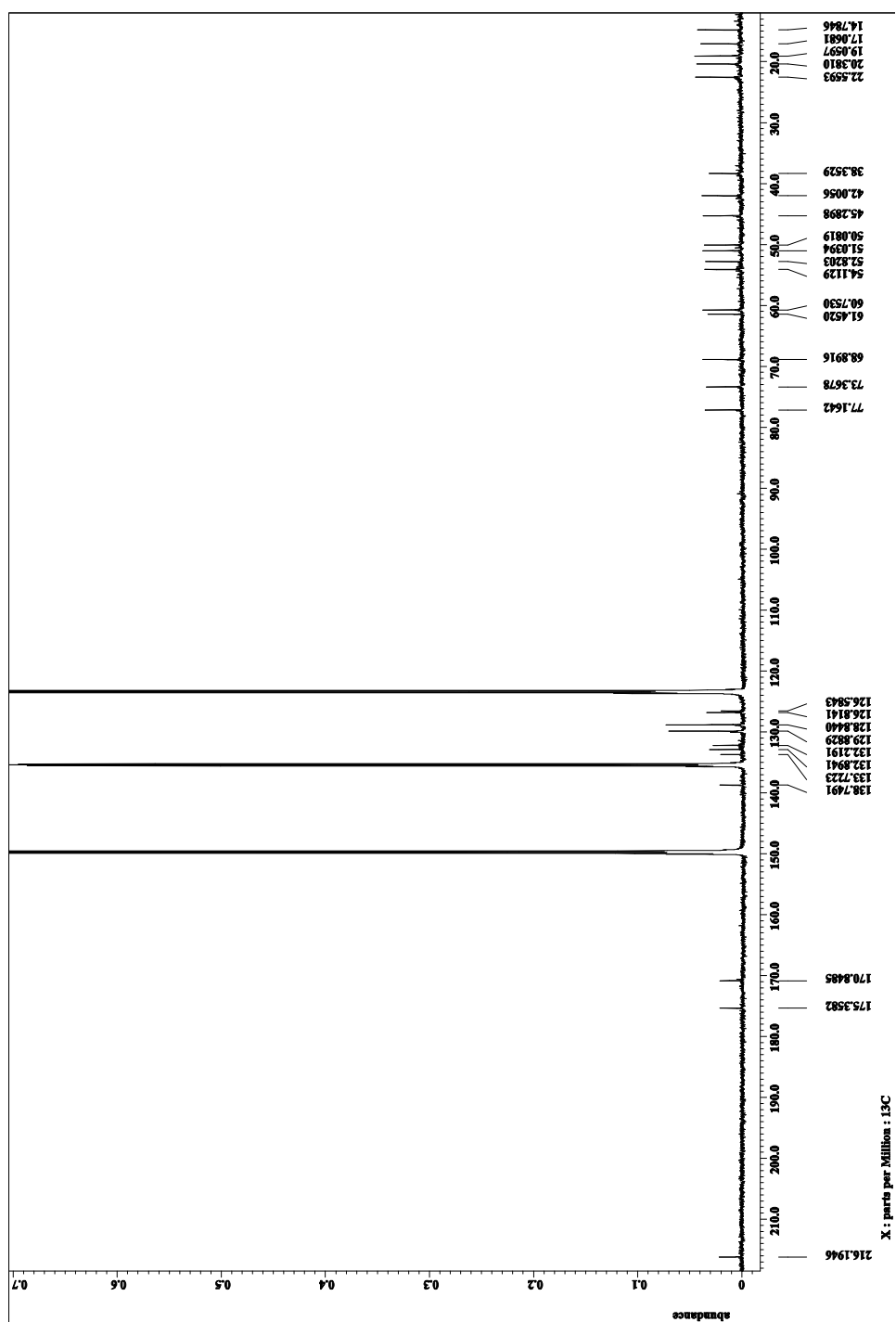


Xylobovatin

Figure 48 Epoxycytochalasins from *X. obovata*

19,20-Epoxycytochalasin C has been isolated previously in Bradford University from *Rosellinia arcuata*³⁷. In this project this compound has been isolated from endophytic *Xylaria* species.

Figure 49 ¹H NMR spectrum of slightly impure 19,20-epoxycytochalasin C

Figure 50 ^{13}C NMR spectrum of 19,20-epoxycytochalasin C

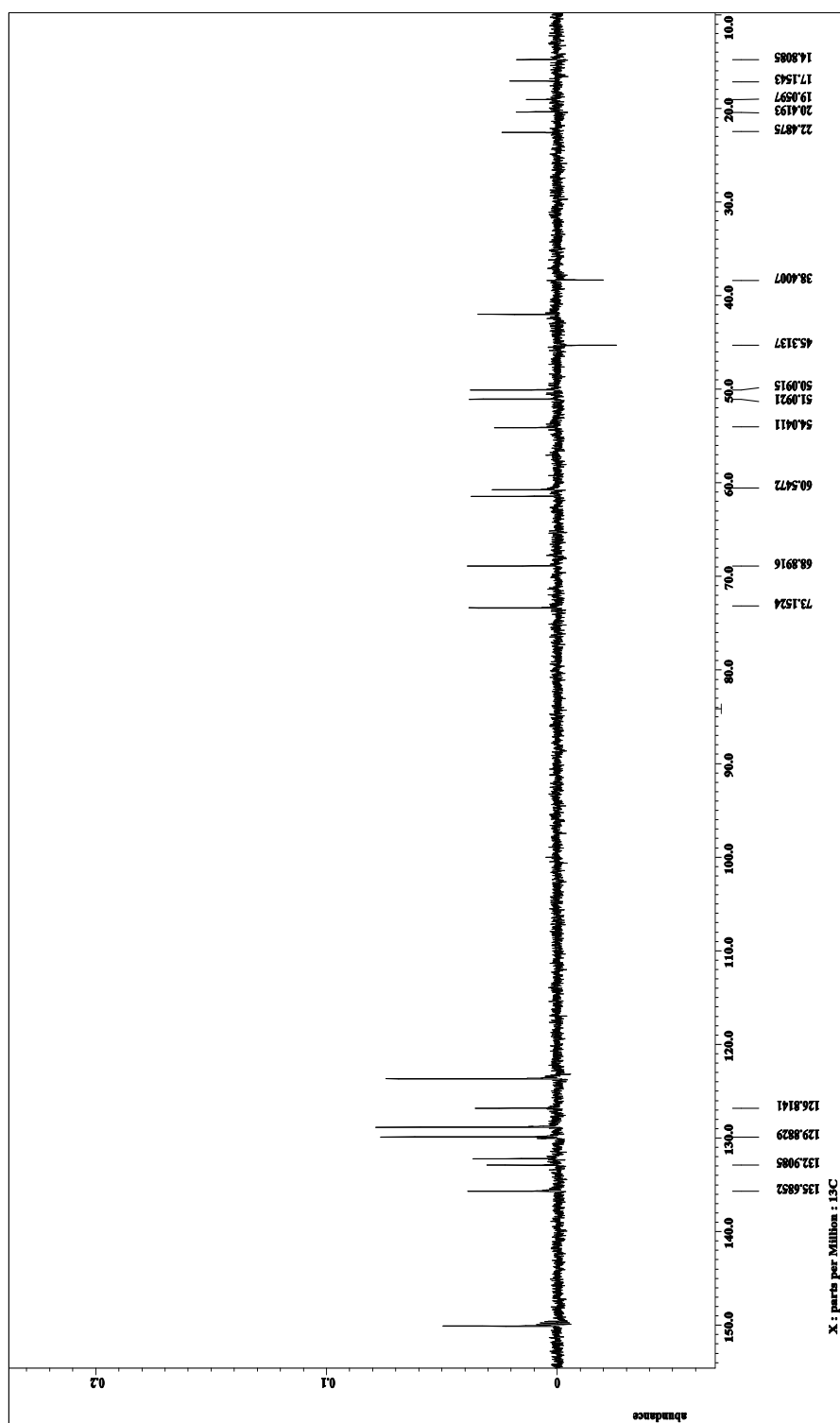


Figure 51 DEPT-135 NMR spectrum of 19,20-epoxycytochalasin C

Chapter 5 Secondary metabolites from the fungus *Engleromyces sinensis*

5.1 *Engleromyces sinensis*

The samples of this fungus, which belongs to the family Hypocreaceae were collected on Snow Mountain at ca 2700 m, Lijiang, Yunnan province in China¹¹⁷. It was classified as *Engleromyces sinensis* **Figure 52**, a species which is highly distributed in Yunnan province, on bamboo stems in Yulong mountain, China. It is the second species in the genus after *Engleromyces goetzei*¹¹⁸.



Figure 52 Fungus *Engleromyces sinensis* as received from China

Engleromyces sinensis is described as new and its distinguishing characteristics are contrasted with those of *Engleromyces goetzei*, which is found in East Africa¹¹⁷. The fungus had yellowish flesh with a hard yellowish –brown exterior crust **Figure 52**. The old herbarium material was hard and horny, but became soft when soaked in water. Morphological examination and a taxonomic study has been reported by Whalley *et al*¹¹⁷ in order to determine the characteristic features.

The hard crust of the fungus was cut into pieces and pulverised using a grinder. The powdered sample (60.5 g) was packed firmly into a soxhlet thimble, which was placed in a soxhlet apparatus. The sample was extracted successively with four different solvents (400 ml of each) for 24 h. The hexane fraction yielded yellowish oil (1.4 g), whilst the chloroform fraction yielded a light brown gum (0.5 g). Ethyl

acetate fraction yielded a light brown gum (43 mg) and methanol fraction yielded a dark brown gum (7.1 g).

The different extracts were then examined for their metabolite profile by TLC. A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. Hexane extract showed a UV active spot R_f 0.9, whilst chloroform extract did not show any UV active spots. Ethyl acetate and methanol extracts showed matching patterns of a single spot under UV Lamp at R_f 0.32. When the TLC plates of both these extracts were sprayed with *p*-nitroaniline spray reagent the spot developed an opaque colouration, suggesting that the ethyl acetate and methanol extracts contained the same component.

5.2 Isolation of secondary metabolites from fungus *Engleromyces sinensis*

5.2.1 Isolation of Engleromycin acetate

A white precipitate formed in the ethyl acetate extract. The white solid (23 mg) was filtered off and recrystallised twice from ethyl acetate to yield colourless needles, mp 265-269 °C, ES $[M+H]^+$ m/z 524, $[\alpha]$ -220° (c 1, in $CHCl_3$). This compound showed an opaque spot when treated with diazotised *p*-nitroaniline spray reagent at R_f 0.32 on TLC. The IR_{ATR} spectrum of this compound showed distinguishable signals: 3748, 3378, 1754 and 1670 cm^{-1} , which could indicate the presence of the following: hydroxyl, amino, carboxylic ester and amide functional groups.

1H NMR spectrum (C_5D_5N , Figure 57) of this compound was indicative of a cytochalasin which showed N-H resonance position at 9.13 in the 1H NMR spectrum. In the ^{13}C NMR and DEPT-135 spectra (C_5D_5N , Figure 58 & 59) an exocyclic methylene signal at δ_C 112.03 and two possible alkene protons at δ_H 5.27 and 5.59 in the 1H NMR spectrum suggested that this cytochalasin (m/z M^+ 523) could be

related to cytochalasin D (m/z M^+ 507) **Figure 53**. The difference of 16 mass unit suggested an additional oxygen atom is present.

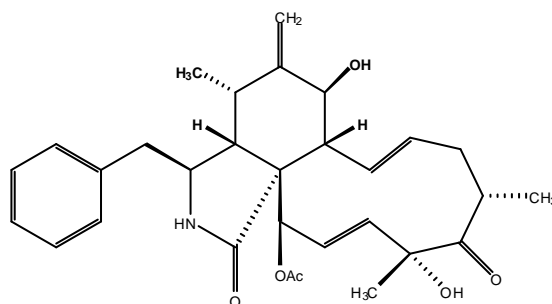


Figure 53 Cytochalasin D

The compound has four methyl groups; which appear as two singlets at δ_H 1.51 and 2.07 and two doublets at δ_H 0.58 and 0.91, which again points to a cytochalasin D related molecule.

The ^{13}C NMR and DEPT-135 spectra of this compound present 28 carbon signals. The signals at δ_C 128.77 and 129.97 are double height of other peaks signifying that these signals are each due to two carbon atoms, to give a total of 30 carbons as in cytochalasin D.

Comparison of the NMR spectral data for cytochalasin D **Chapter 2** and the NMR data of this compound revealed striking changes in the resonances associated with 19,20 region of the molecule as shown in the **Table 17**.

Table 17 Comparison of NMR data in 19,20 region of the two cytochalasins

No	Cytochalasin D		Unknown cytochalasin	
	δ_C	δ_H	δ_C	δ_H
19	132.18	5.59	54.37	3.65
20	133.81	6.83	60.70	4.23

The resonances at δ_{H} 3.65 and 4.23 and δ_{C} 54.37 and 60.70 indicates that the two 19,20- methine protons are attached to oxygen atom bearing carbons. This data confirms the presence of an epoxide at C-19 and C-20 instead of the carbon-carbon double bond present in cytochalasin D.

The presence of the epoxide group at C-19,C-20 and an exocyclic methylene group at C-12 suggests that this compound is the epoxide derivative of cytochalasin D, which is known as 19,20-epoxycytochalasin D³⁰ or engleromycin acetate²⁶ **Figure 54.**

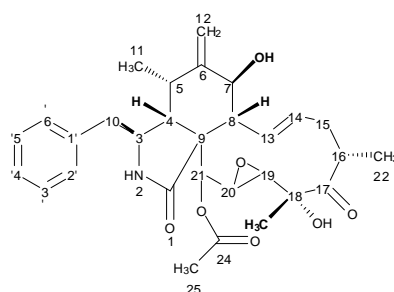


Figure 54 Engleromycin acetate

The chemical shift of the N-H proton of the lactam ring in cytochalasin D appears at δ_{H} 9.08, when pyridine- d_5 is used as the NMR solvent whilst, the N-H resonance of engleromycin acetate is at δ_{H} 9.13. 1D and 2D NMR experiments were used to determine the assignments of the NMR data **Table 18.**

Table 18 ^1H , ^{13}C and DEPT assignments for engleromycin acetate ($\text{C}_5\text{D}_5\text{N}$)

No	δ_{H}	δ_{C}	DEPT
1		175.04	C
2	9.13 (1H, s)	NH	
3	3.46 (1H, s)	53.96	CH
4	2.45 (1H, s)	50.57	CH
5	3.00-3.10 (1H, m)	32.93	CH
6		151.39	C
7	4.32(1H, d, J 10.3 Hz)	71.37	CH
7-OH	4.86 (1H, s)		
8	3.20 (1H, s)	47.33	CH
9		53.66	C
10	2.80-2.89 (2H, m)	45.43	CH_2
11	0.58 (3H, d, J 6.6 Hz)	13.36	CH_3
12a	5.27 (1H, s)	112.03	CH_2
12b	5.59 (1H, s)		
13	5.91-5.96 (1H, m)	132.04	CH
14	6.49-6.56 (1H, m)	132.53	CH
15a	1.91 (1H, d, J 6.5 Hz)	38.19	CH_2
15b	2.71-2.76 (1H, m)		
16	2.77-2.79 (1H, m)	42.46	CH
17		216.37	C
18		77.30	C
19	3.65 (1H, d, J 2.5 Hz)	54.37	CH
20	4.23 (1H, d, J 2.5 Hz)	60.70	CH
21	6.16 (1H, s)	75.11	CH
22	0.91 (3H, d, J 6.5 Hz)	18.95	CH_3
23	1.51 (3H, s)	20.36	CH_3
24		170.62	C
25	2.07 (3H, s)	22.71	CH_3
1'		138.38	C
2',6'	7.20-7.35 (2H, m)	129.97	CH
3',5'	7.20 -7.35 (2H, m)	128.77	CH
4'	7.20-7.35 (1H, m)	126.78	CH

Engleromyces is a genus that belongs to Hypocreaceous fungi¹¹⁷. *Engleromyces*, has two species; *Engleromyces goetzei*¹¹⁷ which grows on the upper stems of the high mountains bamboo in Kenya, East Africa as well as in Yunnan in China. On the other hand, *Engleromyces sinensis*¹¹⁷, the second species in the genus, occurring on bamboo stems in Yunnan and Sichuan Province in China.

Little attention has been paid to the chemical constituents of this inedible fungus. A novel N-containing compound, neoengleromycin **Figure 55** alongside two known cytochalasins; cytochalasin D and 19,20-epoxycytochalasin D were isolated from the fruiting bodies of *Engleromyces goetzei*^{26,27} which was collected from Lijiang, Yunnan province in China.

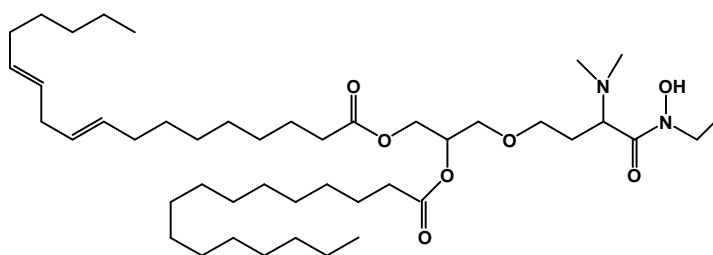


Figure 55 Neoengleromycin

Another group of workers, Pedersen¹¹⁹ *et al* isolated engleromycin; an epoxide of cytochalasin D from the cultures of *Engleromyces goetzei* Hennings collected from Kenya **Figure 56**.

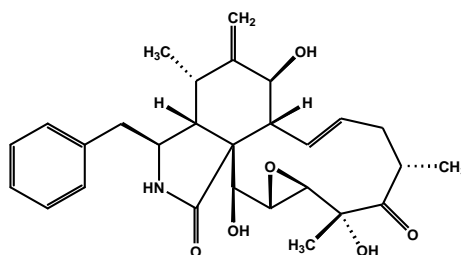
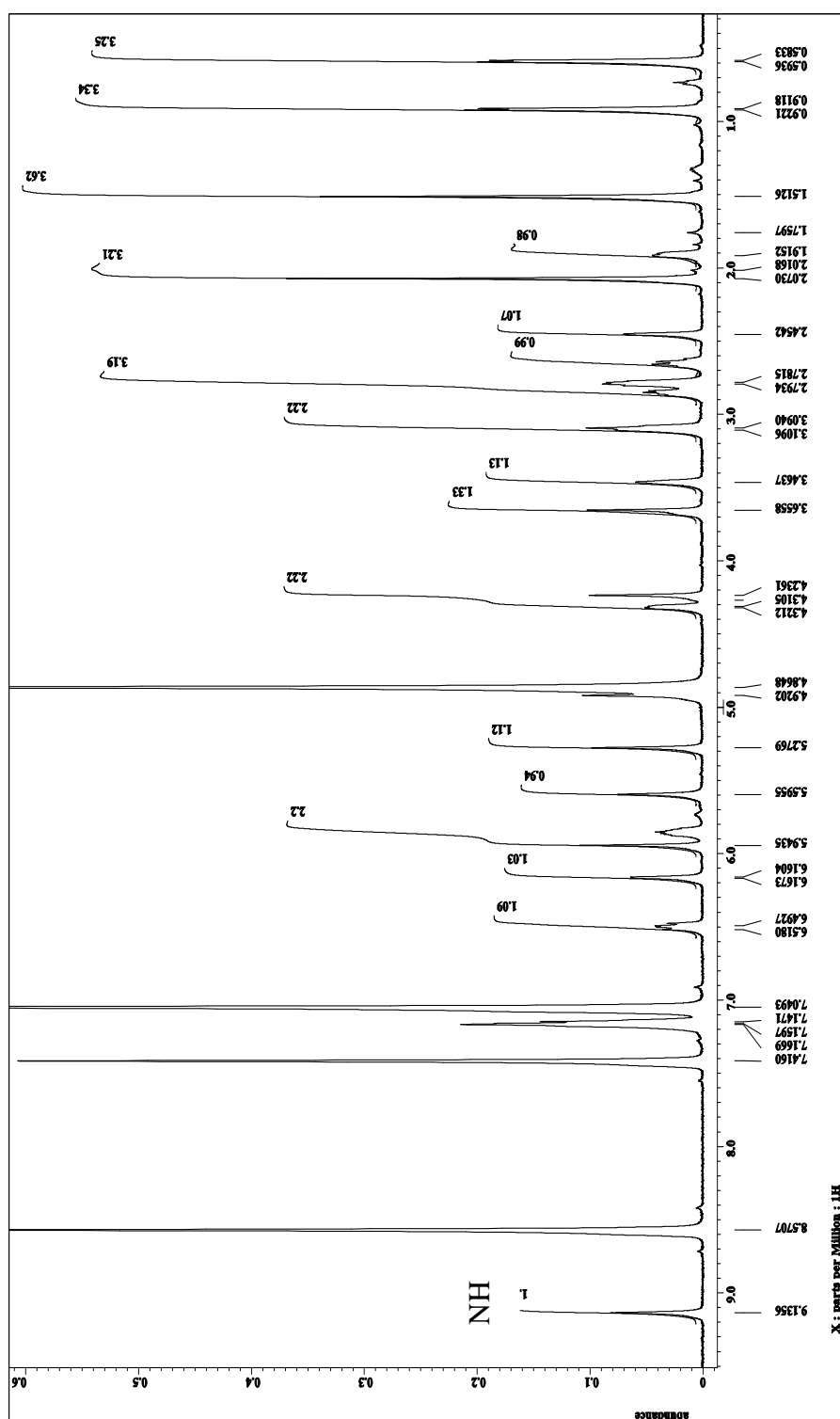
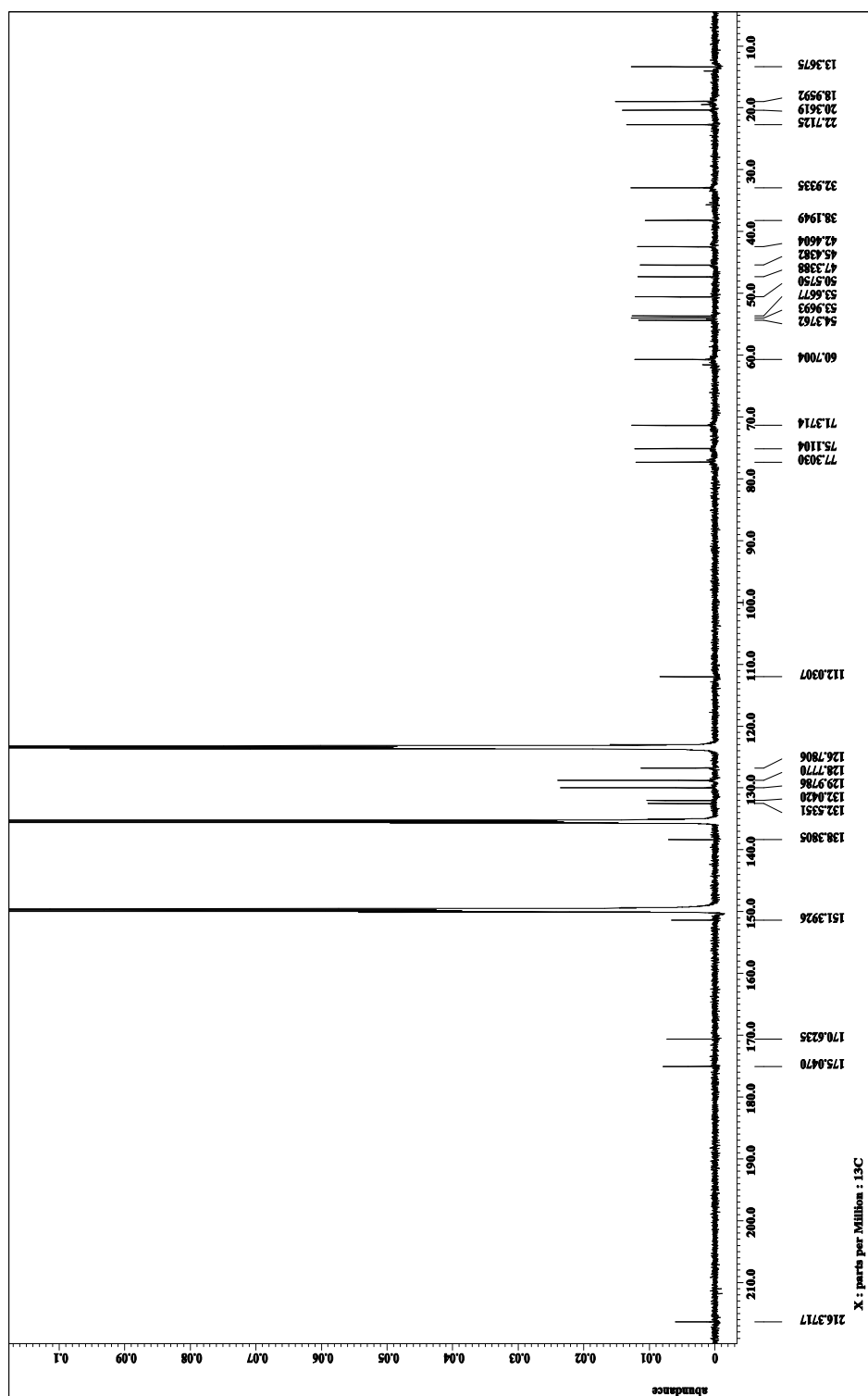


Figure 56 Engleromycin structure

Engleromycin acetate has been isolated previously at Bradford from the cultures of fungus *Rosellinia arcuata*¹²⁰. In this project, engleromycin acetate was isolated from fungus *Engleromyces sinensis*.

The medicinal uses of *Engleromyces sinensis* in China is to treat illnesses due to its antiinflammatory and antimicrobial properties¹¹⁷, whilst *Engleromyces goetzei*¹¹⁷ has been used in traditional African medicine for the treatment of the fever associated with malaria. *Engleromyces goetzei*²⁶, which grows on the bamboo of high mountains is used in the treatment of infection and cancer diseases in the area around Tibet of China including Yunnan and Sichuan Provinces.

Figure 57 ¹H NMR spectrum of engleromycin acetate

Figure 58 ^{13}C NMR spectrum of engleromycin acetate

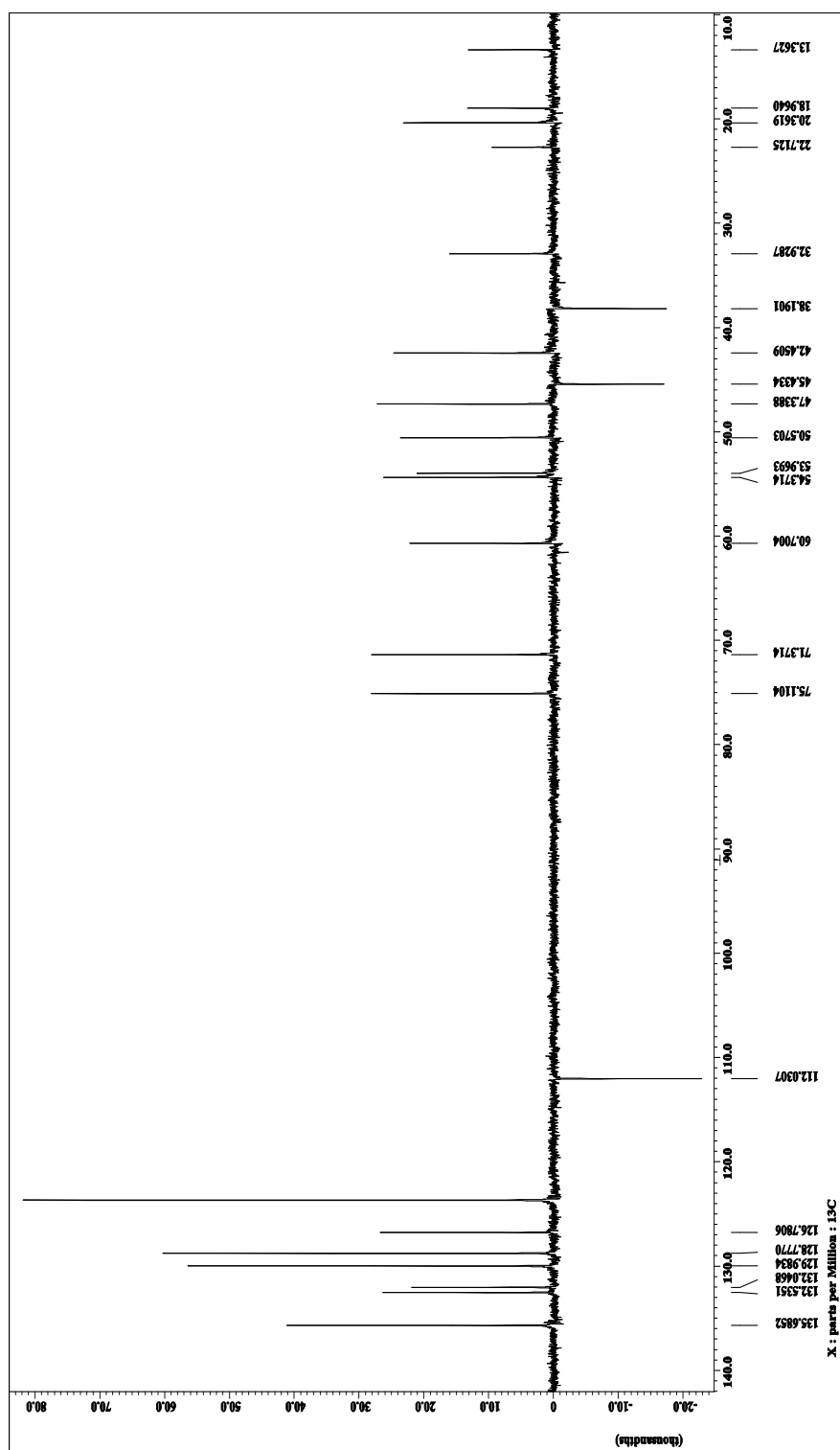


Figure 59 DEPT-135 spectrum of engleromycin acetate

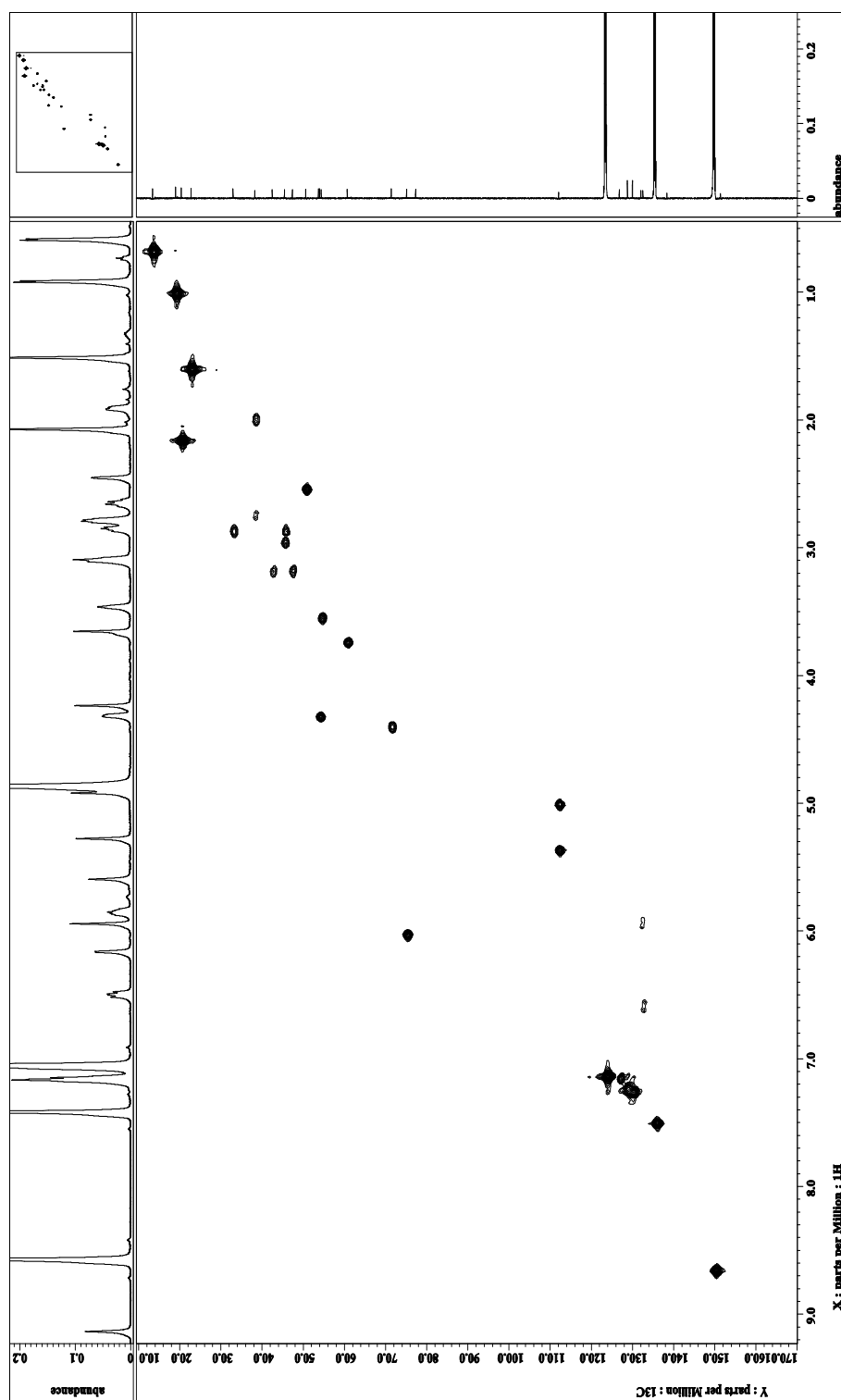


Figure 60 HMQC spectrum of engleromycin acetate

Chapter 6 Secondary metabolites from the fungus *X. polymorpha*

6.1 Fungus *X. polymorpha*

The fungus *X. polymorpha*, which is a plant pathogen, was collected in Thailand. It was received in Petri dishes as shown in **Figure 61**. The front and the back views of the Petri dish revealed a white mycelium.

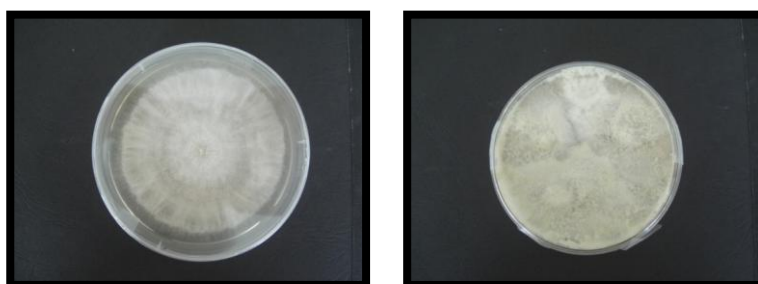


Figure 61 Fungus *X. polymorpha* as received from Thailand

The fungus was static sub-cultured for two weeks into 5 conical flasks (250 ml) containing an aqueous malt extract medium. The fungus developed light brown cylindrical shaped-stromata with unbranched white tips. It produced a white mycelium with a light brown gelatinous underside **Figure 62**.



Figure 62 Fungus *X. polymorpha* in a flask after 2 weeks

The fungus was then cultured in Thompson bottles (15 x 1 L), each bottle containing about (500 ml) of a malt-yeast extract-glucose mixture for 8 weeks. The fungus during the first 4 weeks produced a cotton-like mycelium **Figure 63**.



Figure 63 The mycelium of fungus *X. polymorpha*

The matured cultures were harvested and the mycelia were recovered by filtration through a muslin cloth. The aqueous filtrate was extracted with ethyl acetate using a separating funnel (5 L). The ethyl acetate extracts were combined and dried over anhydrous sodium sulphate. Evaporation of the solvent *in vacuo* yielded the crude extract (2.3 g).

The composition of the crude extract was examined using TLC in different solvent systems; toluene, ethyl acetate and acetic acid (50:49:1), chloroform and methanol (95:5) and petroleum ether (60-80 °C), ethyl acetate and acetic acid (30:70:1). The resultant plates showed that the crude extract contained a single spot with R_f 0.4.

When the TLC plate was sprayed with *p*-nitroaniline spray reagent, it developed an orange colouration. The mycelium contained insignificant quantities of metabolites.

6.2 Isolation of secondary metabolites from fungus *X. polymorpha*

6.2.1 Isolation of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

The component recovered (tubes 20-55) was a yellow oil, which was triturated with ethyl acetate to give a white solid (13 mg), which was recrystallised from the same solvent to yield colourless crystals (7 mg), mp 160-163°C, ES $[M+H]^+$ m/z 281, IR_{ATR} ν_{\max} cm^{-1} 3300, 1774 and 1628. The compound had a R_f value of 0.4 on a silica gel TLC plate eluted with petroleum ether (60-80 °C), ethyl acetate and acetic acid (30:70:1). It showed an intense orange spot with diazotised *p*-nitroaniline spray reagent, suggesting the compound was a phenolic.

The ^1H NMR spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 72) showed 3 singlet methyl signals at δ_{H} 2.28, 2.43 and 3.48. A down field methine proton at δ_{H} 4.79 (1H, dd, J 6.6, 1.1 Hz) indicating that this proton could be attached to carbon attached to an oxygen atom. A singlet signal for a methine proton in the aromatic region at δ_{H} 7.49. Two alkene protons, as indicated by the 16.2 Hz *trans* coupling constant at δ_{H} 7.60 (1H, dd, J 16.2, 6.6 Hz) and 7.75 (1H, dd, J 16.2, 1.1 Hz). A strongly chelated-OH at δ_{H} 14.06 suggesting that the hydroxyl group is intramolecularly hydrogen bonded to the carbonyl oxygen.

The ^{13}C NMR spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 73) showed 14 signals comprised of three methyl carbons at δ_{C} 17.08, 26.08 and 56.95. The methyl group at δ_{C} 56.95 suggests it is attached to oxygen atom. Four methine carbons at δ_{C} 83.58, 124.07, 129.91 and 132.35. Five quaternary carbons in the aromatic region which occur at δ_{C} 112.35, 113.02, 116.80, 162.27 and 162.70. The quaternary carbon at δ_{C} 203.37 is a typical carbonyl of a ketone. The mass spectrum, ^1H and ^{13}C NMR suggested a molecular formula of $\text{C}_{14}\text{H}_{16}\text{O}_6$.

The ^1H - ^1H COSY spectrum ($\text{C}_5\text{D}_5\text{N}$, Figure 75) showed a single spin system. The doublet of doublet methine proton at δ_{H} 4.79, showed a connectivity to the alkene protons at δ_{H} 7.60, which in turn is *trans* coupled (J 16.2 Hz) to the one proton doublet of doublets at δ_{H} 7.75, which gives sub-unit A **Figure 64**.

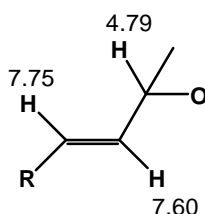


Figure 64 Sub-unit A

The lack of proton coupling information meant that the HMBC spectrum, in association with the HMQC spectrum, played a pivotal role in establishing a structural formula. The proton signal at δ_{H} 4.79 showed 4 long range correlations, which led to sub-unit B **Figure 65**.

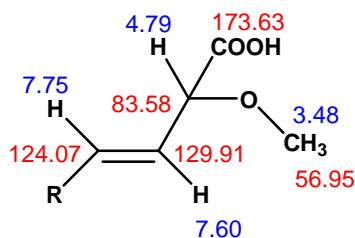


Figure 65 Sub-unit B

The molecular formula of $\text{C}_{14}\text{H}_{16}\text{O}_6$ indicated an index of hydrogen deficiency (IHD) of 7 and sub-unit B only contains two degrees of unsaturation. Thus the partial structure that remains has an (IHD) of 5, which given the presence of an acetyl group (3 proton singlet at δ_{H} 2.43) strongly indicated the presence of a benzene ring; probably penta-substituted (1 acetyl, 2 hydroxyl, 1 methyl and Sub-unit-B).

The downfield singlet at δ_H 14.06 was strongly indicative of intramolecular hydrogen bonding and led to sub-unit C **Figure 66**.

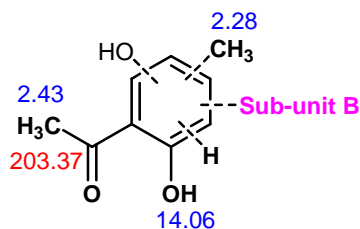


Figure 66 Sub-unit C

The positioning of the substituents was established by careful analysis of the HMQC and HMBC 2D NMR data, which gave sub-unit D **Figure 67**.

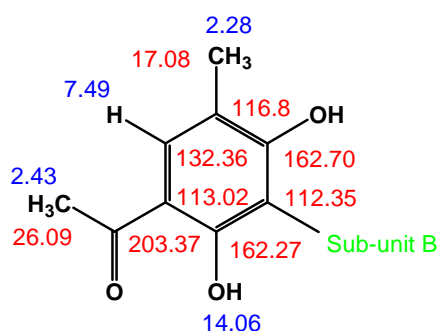


Figure 67 Sub-unit D

Fusion of sub-units B and D led to the structure of this metabolite; (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid **Figure 68**.

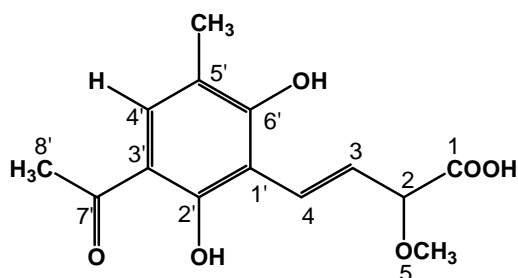


Figure 68 (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid structure

The NMR assignments for the compound, which were established using 1D and 2D NMR spectral experiments are presented in **Table 19 & 20**.

Table 19 ^1H and ^{13}C NMR data for (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid ($\text{C}_5\text{D}_5\text{N}$)

No	δ_{H}	δ_{C}	DEPT
1		173.63	C
2	4.79 (1H, dd, J 6.6, 1.1 Hz)	83.58	CH
3	7.60 (1H, dd, J 16.2, 6.6 Hz)	129.91	CH
4	7.75 (1H, dd, J 16.2, 1.1 Hz)	124.07	CH
5	3.48 (3H, s)	56.95	CH_3
1'		112.35	C
2'		162.27	C
3'		113.02	C
4'	7.49 (1H, s)	132.35	CH
5'		116.80	C
6'		162.70	C
7'		203.37	C
8'	2.43 (3H, s)	26.08	CH_3
9'	2.28 (3H, s)	17.08	CH_3
2'-OH	14.06 (1H, s)		

Table 20 HMBC NMR data for (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid ($\text{C}_5\text{D}_5\text{N}$)

No	δ_{H}	δ_{C} $^n\text{J}_{\text{H-C}}$ correlation ex HMBC
2	4.79 (1H, dd, J 6.6, 1.1 Hz)	56.95, 124.07, 129.91, 173.63
3	7.60 (1H, dd, J 16.2, 6.6 Hz)	83.58, 112.35
4	7.75 (1H, dd, J 16.2, 1.1 Hz)	83.58, 162.27, 162.70
5	3.48 (3H, s)	83.58
4'	7.49 (1H, s)	17.08, 162.27, 162.70, 203.37
8'	2.43 (3H, s)	113.02, 132.35, 203.37
9'	2.28 (3H, s)	132.35, 162.70, 113.02
2'-OH	14.06 (1H, s)	113.02, 162.70

A literature search revealed that the isolation of this compound has been reported previously³⁴ by Edwards *et al* in 1995. They isolated Globoscin (**1**) and Globoscinic acid (**2**) **Figure 69** from the culture media of the fungus *X. globosa*, which was collected from a forest in the Cameron. During the course of that work they found that Globoscin (**1**) rearranged slowly in pyridine to give (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid. Since deuteropyridine was used as the NMR solvent in this work, it is highly likely that the product identified is in fact also a rearrangement product.

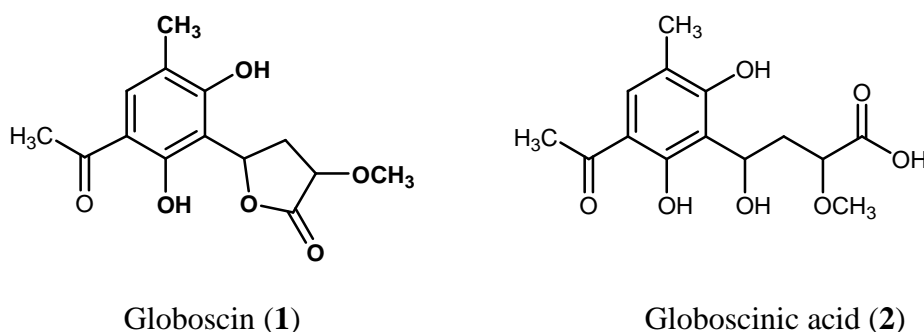


Figure 69 Secondary metabolites from *X. globosa* and *X. obovata*

Xylaria polymorpha, a hard wood-colonizing Ascomycete, commonly known as dead man's fingers, is a plant pathogen¹²¹. It is a common inhabitant of forest and woodland areas, usually growing from the bases of rotting or injured tree stumps and decaying wood. It is distributed worldwide in tropical areas, produces various secondary metabolites that exhibit potential biological activities.

Three isopimarane diterpene glycosides; 16- α -D-mannopyranosyloxyisopimar-7-en-19-oic-acid (**3**), 15-hydroxy-16- α -D-mannopyranosyloxyisopimar-7-en-19-oic-acid (**4**) and 16- α -D-glucopyranosyloxyisopimar-7-en-19-oic-acid (**5**) **Figure 70** , have been isolated from the fruiting bodies of the fungus *Xylaria polymorpha*¹²², which was fermented on an unpolished rice media.

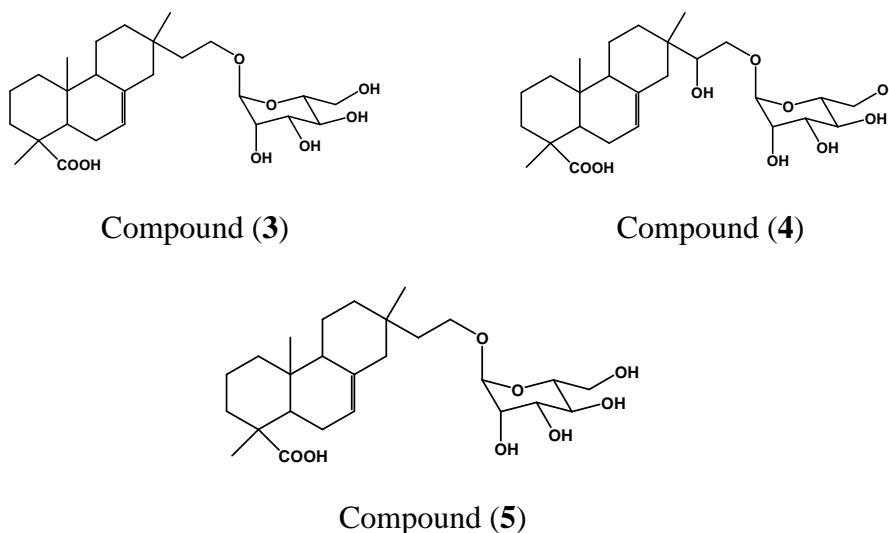
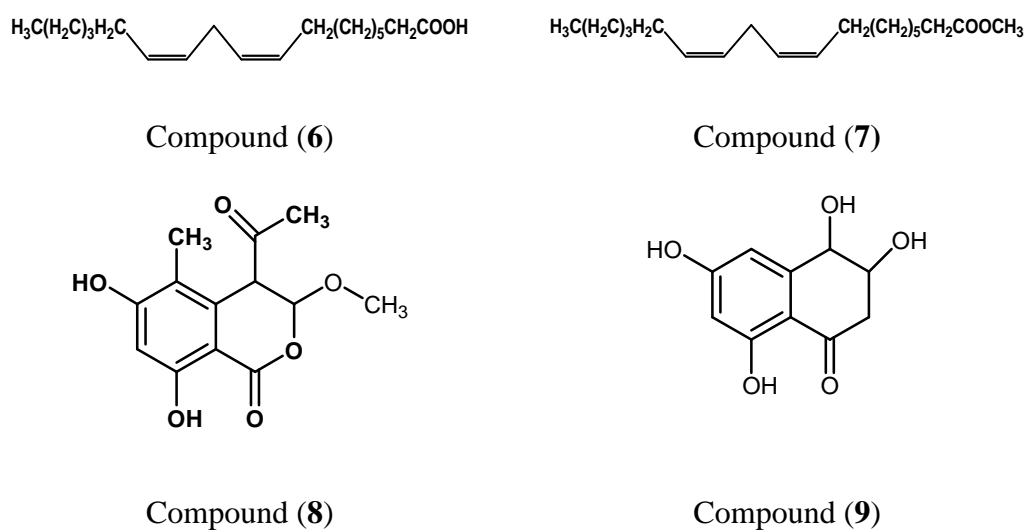


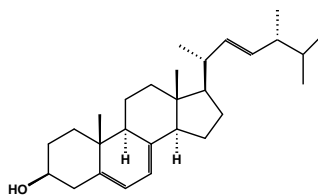
Figure 70 Secondary metabolites from *X. polymorpha*

The new diterpene derivatives showed cytotoxicity against human cancer cell lines and exhibited IC_{50} values ranging from 71-607 μM .

Jang *et al*¹²³ have identified the chemical constituents of the fruiting bodies of *Xylaria polymorpha*. Linoleic acid (6), linoleic acid methyl ester (7), 4-acetyl-3,4-dihydro-6,8-dihydroxy-3-methoxy-5-methyl-1*H*-2benzopyran-1-one(8),4-hydroxyscytalone (9) and ergosterol (10) were isolated from its methanolic extract

Figure 71.





Compound (10)

Figure 71 Secondary metabolites from *X. polymorpha*

Laccase¹²⁴ is a ligninolytic oxidoreductase enzyme produced by the fungus *Xylaria polymorpha*, which was fermented on tomato juice complex medium. The enzyme has a potential ability to modify non-phenolic lignin structures in the presence of suitable mediators. Also, it may be involved in the melanization process (formation of melanin in wood). Hacıoglu *et al*¹²¹ have examined the antimicrobial potential of the ethanolic extracts obtained from the macro-fungus *Xylaria polymorpha* using agar disc diffusion assays. The fungus showed a significant antimicrobial activity against Gram-positive and Gram-negative bacteria as well as the yeast cultures.

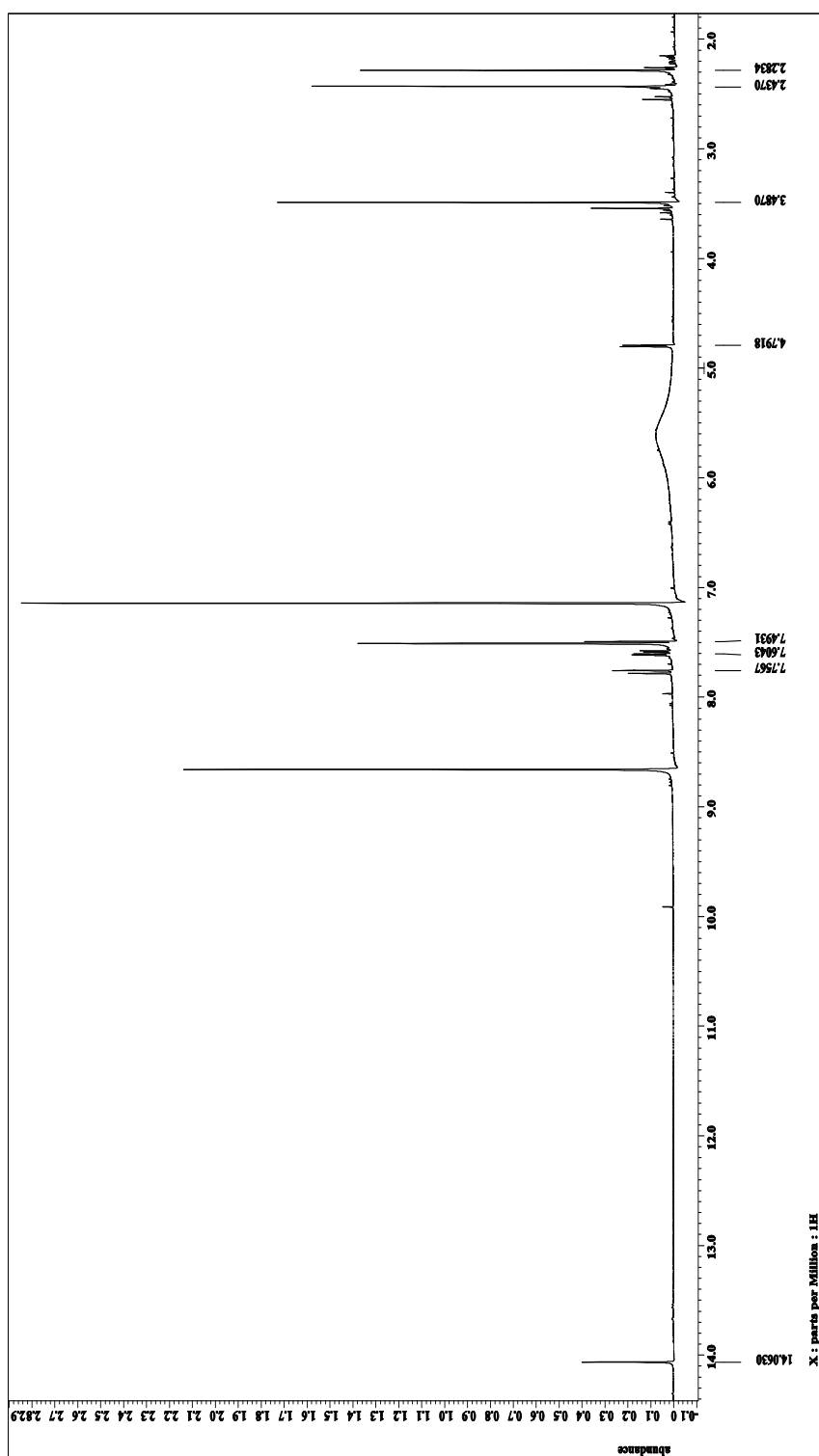


Figure 72 ^1H NMR spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

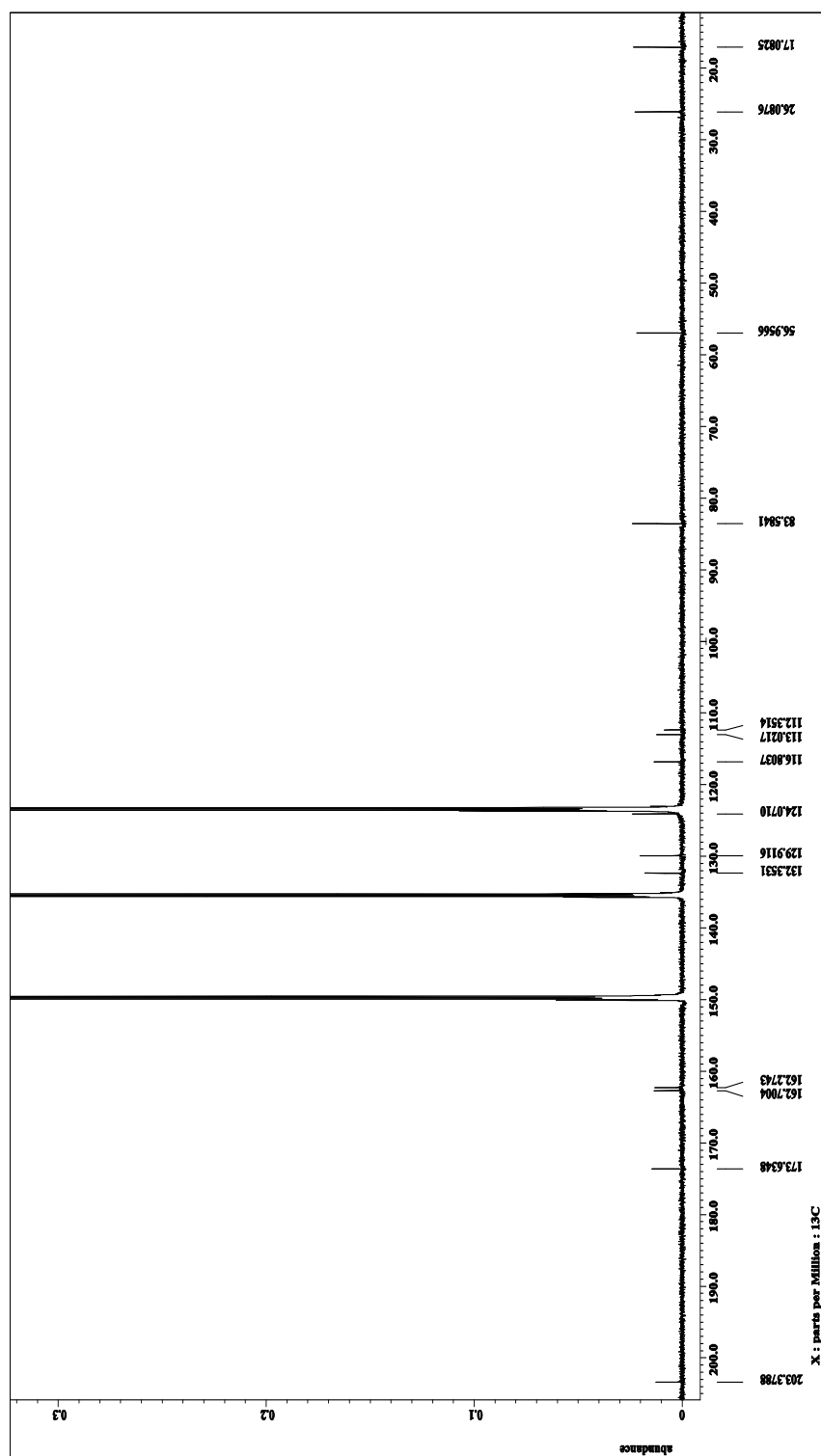


Figure 73 ^{13}C NMR spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methoxyphenyl)-2-methoxybut-3-enoic acid

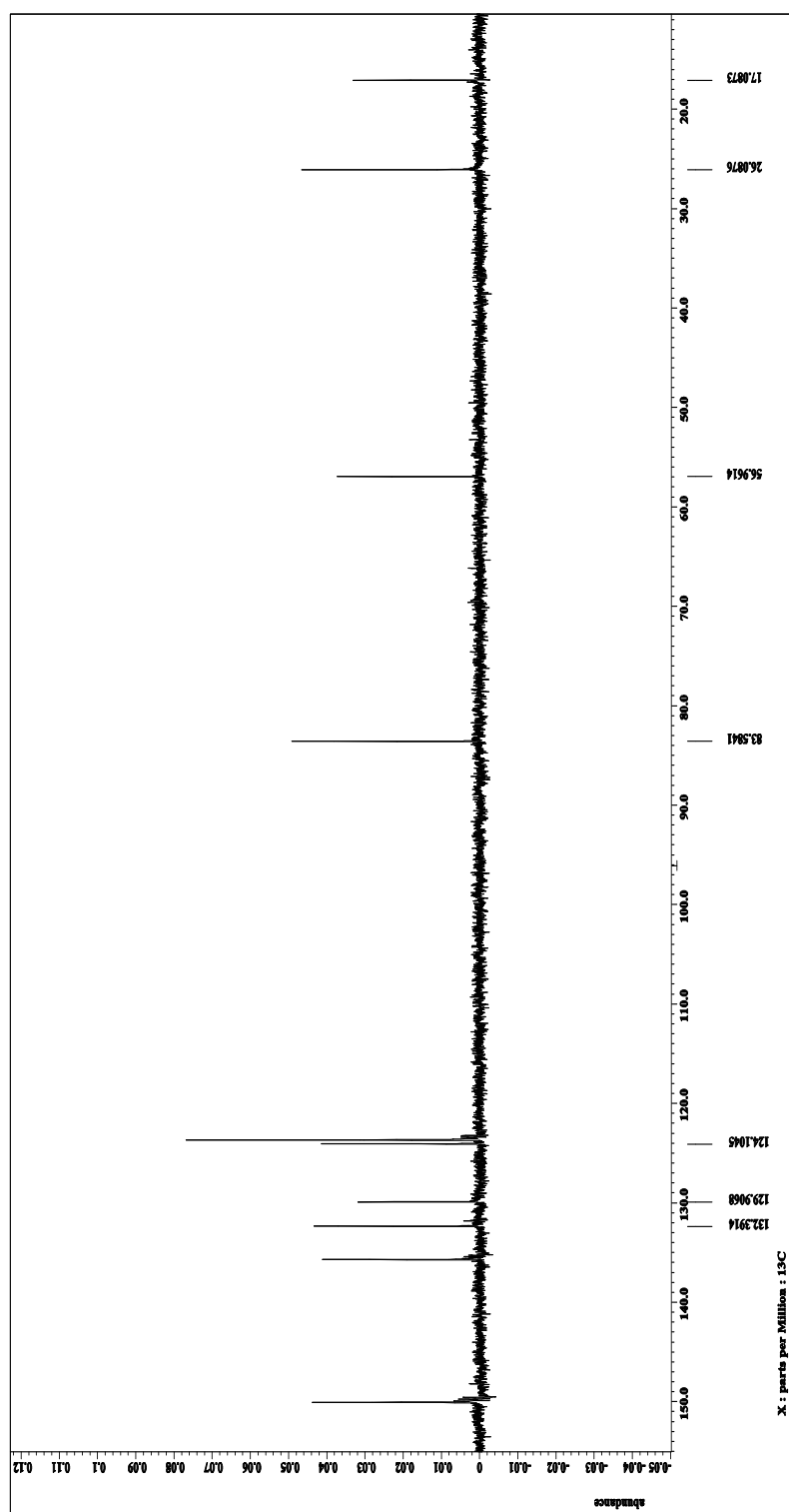


Figure 74 DEPT-135 spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

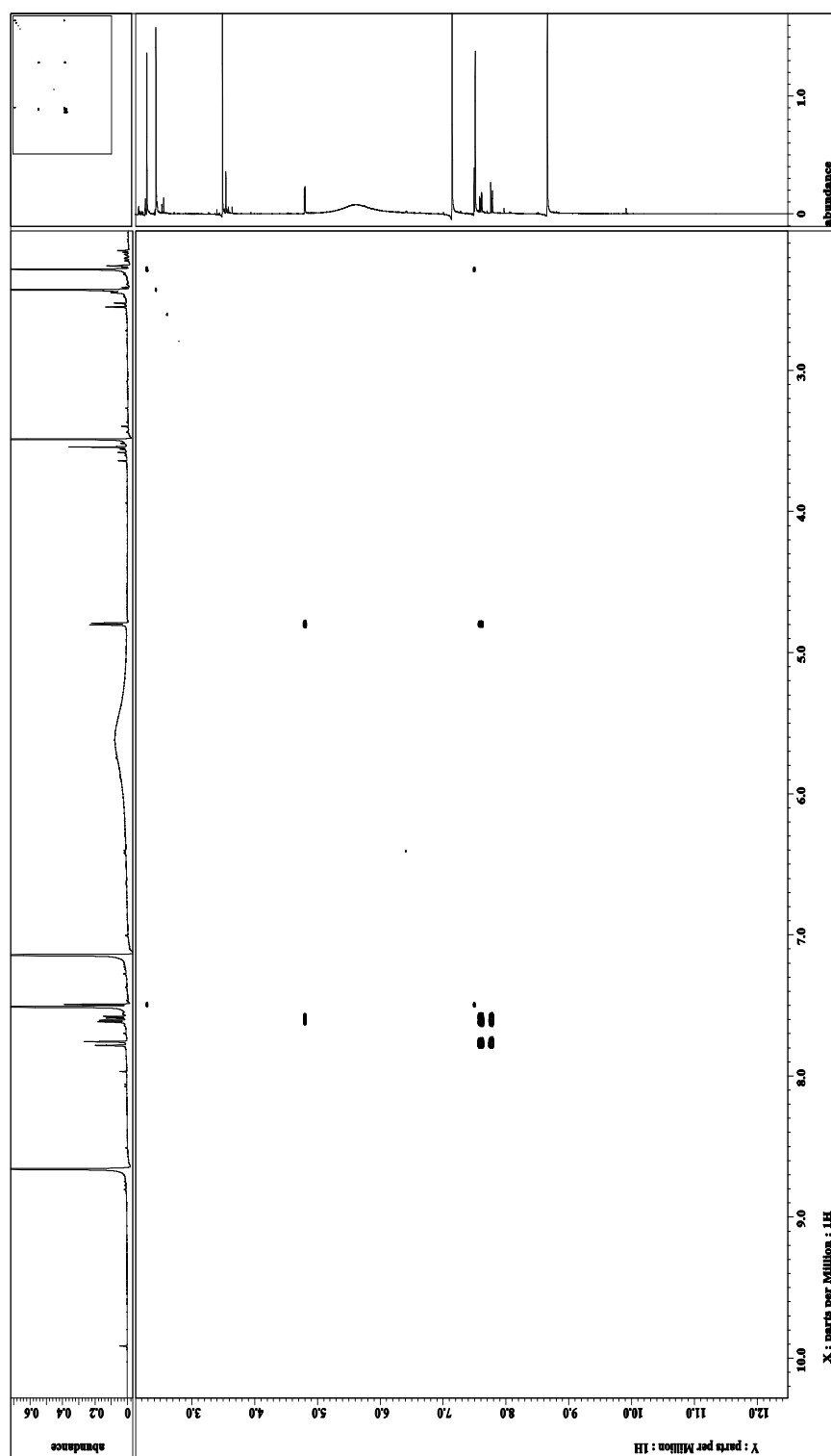


Figure 75 ^1H - ^1H COSY spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methoxyphenyl)-2-methoxybut-3-enoic acid

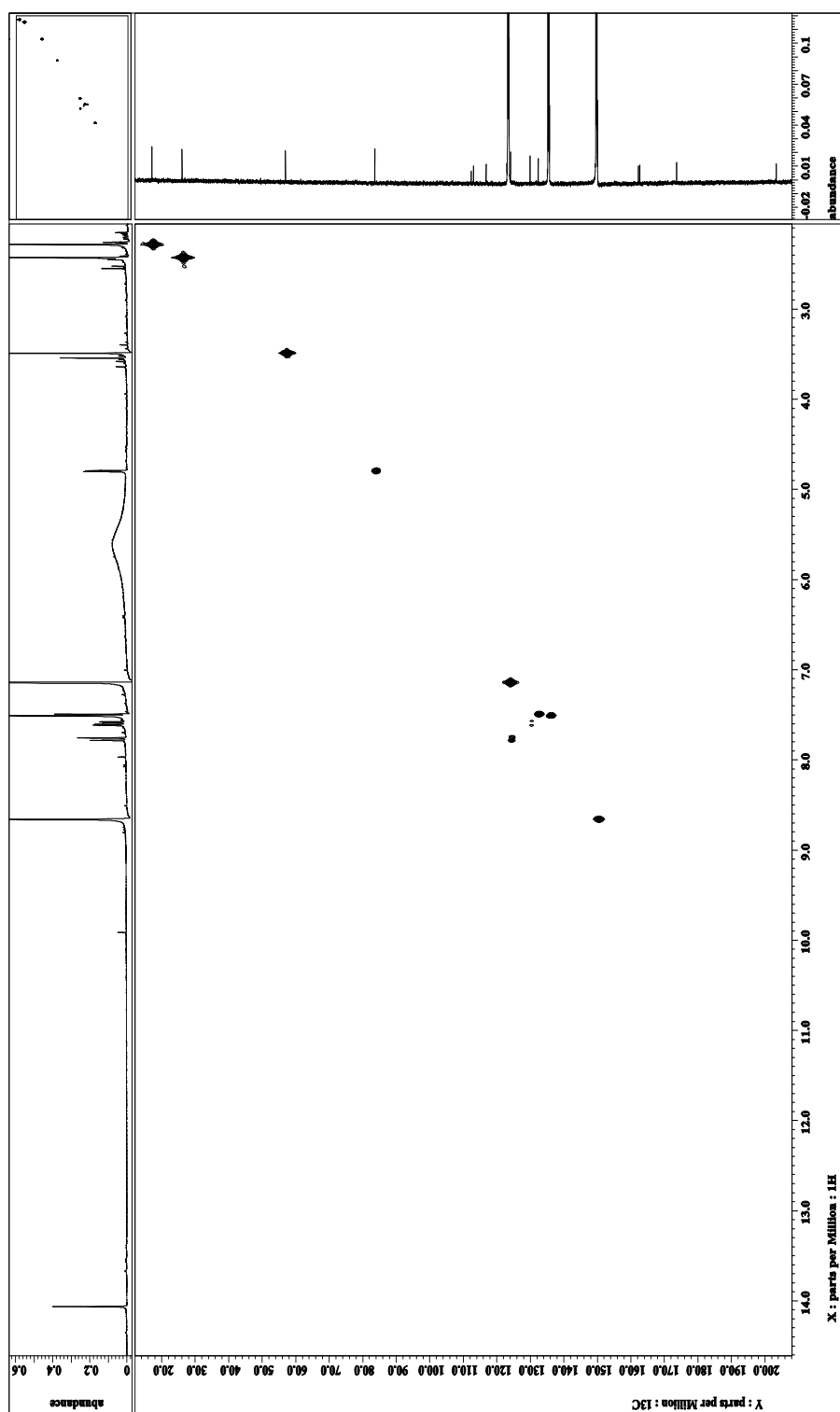


Figure 76 HMQC spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

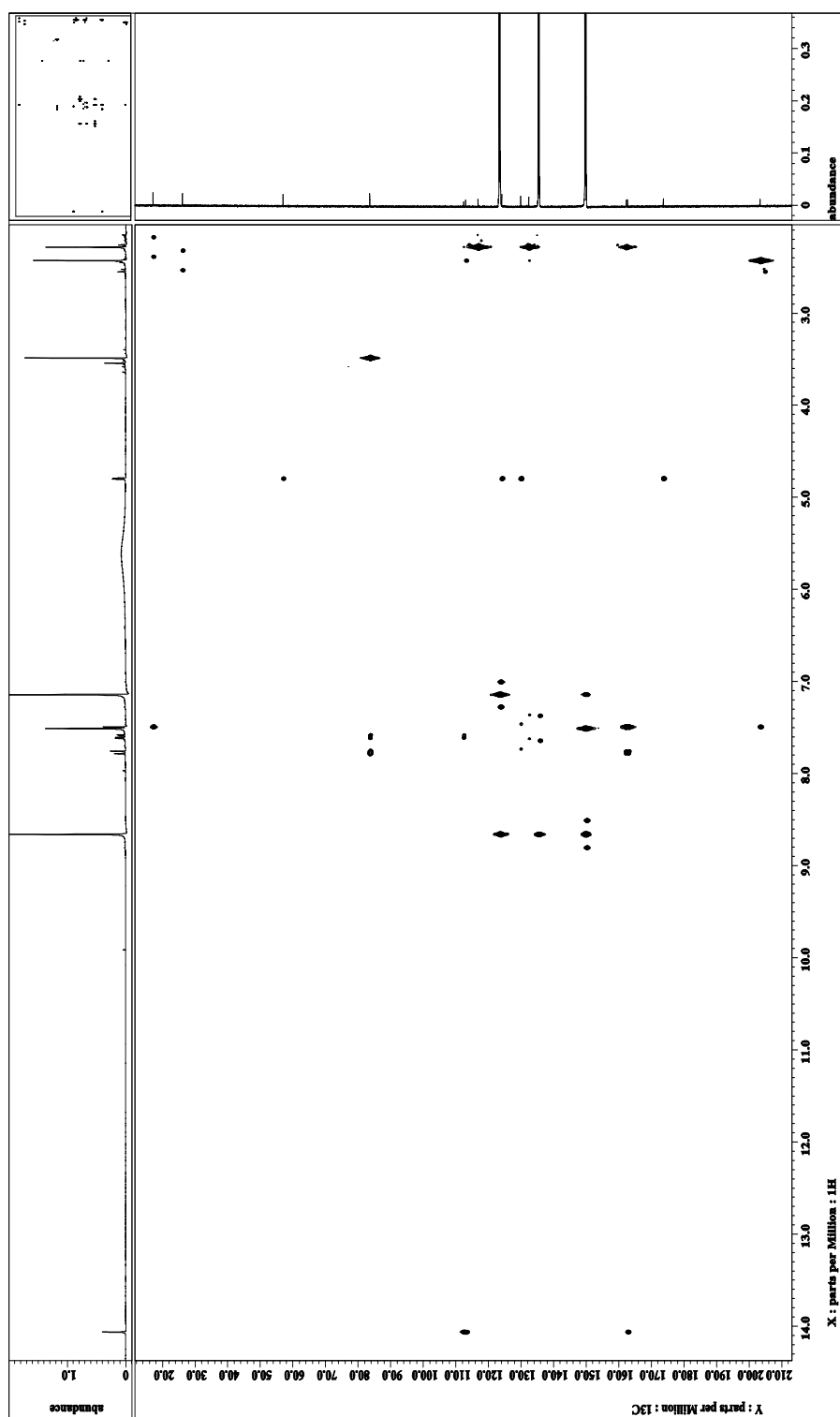


Figure 77 HMBC spectrum of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

Chapter 7 Experimental Work

7.1 Culturing of Fungi

7.1.1 Culture Room

The fungi used in this project were cultured in a special room under specific conditions. The culture room was equipped with the following essentials:

- An autoclave for sterilising the culture media before inoculation.
- A Bunsen burner to sterilise glasses and tools such as spatulas used during the culturing process.
- A thermostatic heating system to keep the culture room at stable temperature suitable for fungi growing.
- A thermometer to monitor room temperature.

7.1.2 Glassware inside the culture room

- Thompson bottles (1 L) and (2 L) to grow the fungi on a large scale.
- Conical flasks (250 ml) to grow the fungi on a small scale.
- Sample tubes with a screw cap to save master cultures of the fungi on agar slopes.

7.1.3 Sterilisation

Sterilisation is the process that is used to kill microorganisms such as yeast, bacteria, fungi and viruses on a surface, equipment and the biological culture media. This process was achieved using a chemical detergent and heating under elevated temperature and pressure as follows:

- First of all, the conical flasks and Thompson bottles were cleaned with water and a biocleaner detergent (2.5 % solution of Microcleanse from Teknon) using a cleaning brush.

- After preparation of the culture medium, a Priorclave was used to sterilise the flasks and Thompson bottles containing the culture medium. The Priorclave uses steam at 121°C for 30 min. The process is completely automated in this modern autoclave **Figure 78**.



Figure 78 The Priorclave used in sterilisation

7.1.4 Master culture

Master cultures of the fungi were kept in 20 ml glass screw-capped tubes. They were prepared as follows:

- An aqueous suspension of 3% malt and 2% bio-agar was mixed with heating.
- 20 ml sample tubes with cap were filled to approximately half their volumes with the prepared suspension.
- The tubes and contents were sterilised by the Priorclave.
- The tubes were then reheated again to 100 °C for 5 min and set in a slope mode so the culture medium solidifies sideways in the tubes.
- Finally the tubes were inoculated with the fungus samples and labelled with code and date.

- For each fungus 4-6 master cultures were prepared. The master cultures are used to sub-culture the fungi into conical flasks, which can be used to reform the master cultures, if required.



Figure 79 Master cultures of different fungi

7.1.5 Sub-culturing in conical flasks

Each fungus was sub-cultured into four conical flasks (250 ml each) for 2-3 weeks before they were transferred into Thompson bottles. The conical flasks provide sufficient material for large scale culturing. They also give information about the growth pattern of the fungi. The content of each conical flask can be used to inoculate 7-10 Thompson bottles of (1 L or 2 L) capacity.



Figure 80 Sub-culture in conical flask

7.1.6 Thompson bottles

Thompson bottles (1 L or 2 L) were used to culture the fungi for large-scale work as follows:

The mycelium of the fungus in the conical flask was crushed into small pieces using a sterilised spatula.

- A small sample of material from the conical flask was transferred into each Thompson bottle, which contained sterilised culture medium.
- Each set of Thompson bottles was labelled with the fungus code and culturing date and kept on sloped shelves especially constructed for this purpose.
- The Thompson bottles were observed for 8 weeks and any contaminated bottle was discarded. The culture room was kept dark at 25-27 °C. The culturing process can be repeated several times as required using the master culture **Figure 81**, shows the steps in the culturing process.

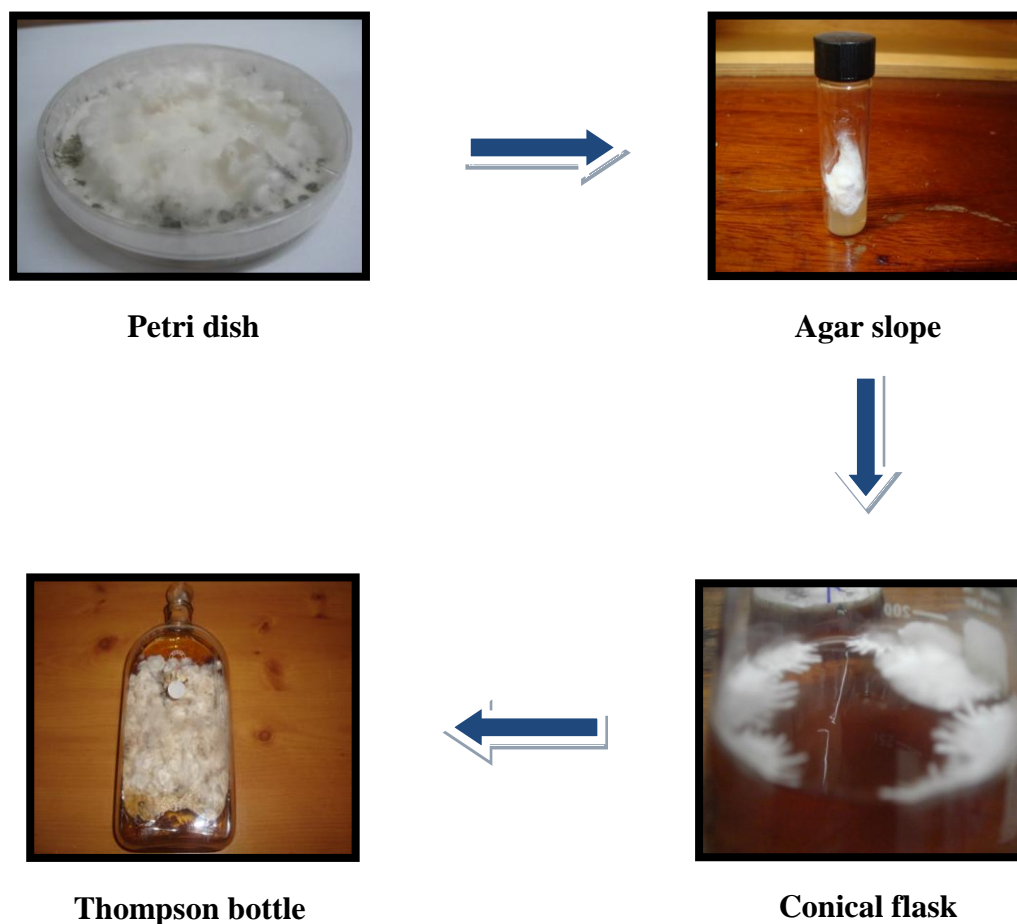


Figure 81 Culturing process steps

7.2 Extraction process

After 8 weeks the contents of the Thompson bottles were combined and the mycelia were separated by filtration through a muslin cloth. The mycelia were air-dried. The medium was extracted in batches of 2.5 L using ethyl acetate (3 x 700 ml) in a separating funnel (5 L). Each time the separating funnel was shaken gently for a few minutes and then left to settle until two distinct layers formed. The upper layer (ethyl acetate extract) was separated and then dried over anhydrous sodium sulphate for 30 min. The ethyl acetate was then removed *in vacuo* using a rotary evaporator. Finally the crude yield of the metabolites was determined.

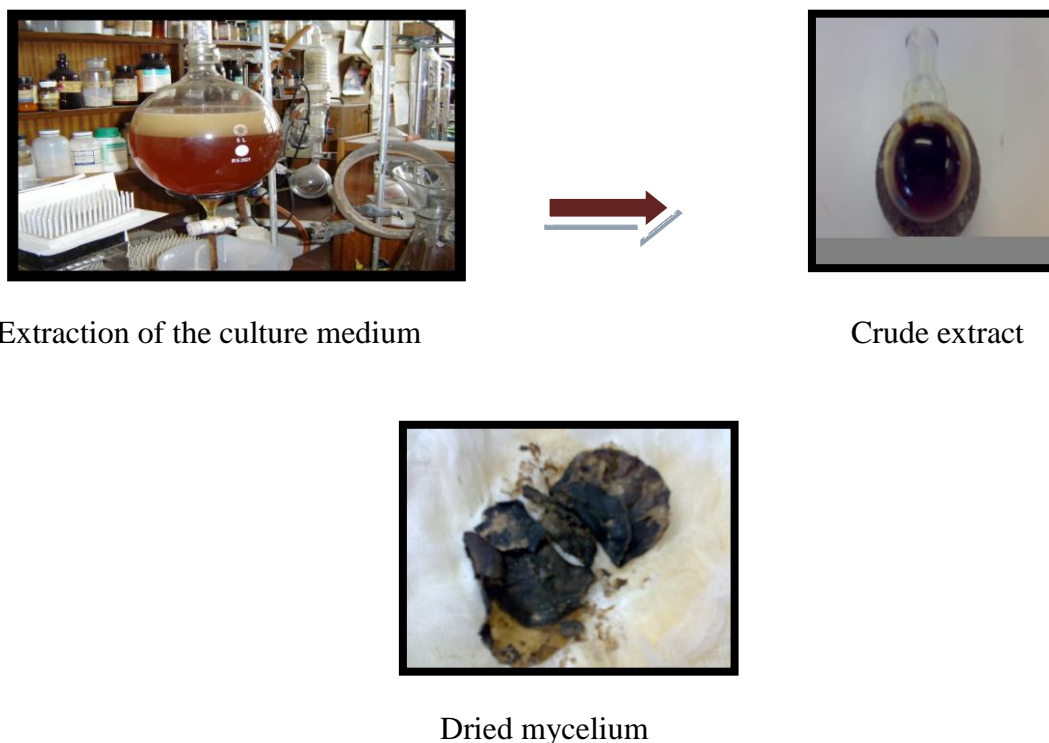


Figure 82 Extraction process of the culture medium

7.3 Chromatography process

Merck Kieselgel 60, GPF254 silica gel was used for TLC and PTLC.

TLC plates (20 cm x 20 cm) were prepared by mixing (200 g) of the silica gel with (480 ml) distilled water in a round-bottomed flask (2 L). The mixture was swirled to make a homogenous suspension and left to stand for 1h. The suspension was spread on a clean glass plate and left to air-dry for 24 h at room temperature.

PTLC plates (20 cm x 80 cm) were prepared by mixing Merck Kieselgel 60, GPF254 silica gel (100 g) with (270 ml) distilled water in a beaker (1 L) and left to settle 45 min before spreading on a clean glass plate. The plate was left to air-dry for 24 h at room temperature. Merck Kieselgel 60, 0.063-0.200 mm pore diameter was used for column chromatography; unless otherwise stated. The size of the columns and the solvent systems used in each case were specified in context.

7.4 Spray reagents

- Diazotised *p*-nitroaniline spray reagent was prepared as follows:

A few drops of 5% sodium nitrite solution (described below) were added to 10% *p*-nitroaniline solution (50 ml) until the yellow colour of the solution disappeared. Then sufficient quantity of 10% aqueous sodium carbonate solution was added until the yellow colour of the solution appeared again.

(The 5% solution of *p*-nitroaniline was prepared by dissolving 7.0 g of *p*-nitroaniline crystals in concentrated hydrochloric acid (90 ml) and then diluted to 1 L.

- Anisaldehyde spray reagent was prepared as follows:

4-Methoxybenzaldehyde (1.0 ml) and concentrated sulphuric acid (1.0 ml) were added to glacial acetic acid (98.0 ml).

7.5 Crystallisation

The solvent conditions used for crystallisation of each pure compound, with single or mixture of solvents, is specified in each instance.

7.6 Physical properties

- Melting points were measured using a Kofler hot-stage apparatus and are uncorrected.
- IR spectra were obtained using a Perkin-Elmer spectrum 100 ATR-IR Spectrometer.
- ESI Mass spectra were determined using a Micromass ZMD (Single Quad) or a Micromass Quattro Ultima (Triple Quad).
- Optical rotations were measured using a Perkin Elmer 141 Polarimeter equipped with a cell of path length 1.0 dm.

- ^1H and ^{13}C and 2D NMR spectra were obtained on a JEOL ECA 600 NMR Spectrometer.
- Carbon atom types were determined by employing a combination of ^{13}C NMR broad-band proton-decoupled spectra, and Distortionless Enhancement by Polarisation Transfer (DEPT) experiments.
- Precise assignments were established by employing a combination of 1D and 2D NMR experiments.
- ^1H - ^1H correlations by double quantum-filtered COSY.
- ^1H - ^{13}C $^1J_{\text{CH}}$ correlations by HMQC pulse sequence.
- ^1H - ^{13}C $^nJ_{\text{CH}}$ correlations by HMBC pulse sequence.

7.7 Secondary metabolites from fungus B127R culture medium

Five endophytic fungi **B231b**, **B127R**, **A116**, **B115** and **B338** were collected in Southern Thailand from palm tree leaves *Borassodendron machadonis* and classified as *Xylaria* endophytes were static sub-cultured on an aqueous solution of malt extract into 10 conical flasks (250 ml) two for each fungus for 2 weeks. Fungus **B231b** and **B127R** grew with a broad white mycelium, whereas fungus **A116** produced black cylindrical shaped-stromata with a black base on a white mycelium. Fungus **B115** developed brown fruiting bodies after 10 days, which were visible on a white mycelium, whilst fungus **B338** produced a black mycelium **Figure 83**. Each endophyte was then transferred into Thompson bottles (10 x 2 L) and allowed to grow on a malt extract-glucose mixture for 8 weeks. Master cultures of these endophytes were made for future reference. The matured cultures were harvested and the mycelia were recovered by filtration through a muslin cloth. The respective aqueous filtrates were extracted into batches of (2.5 L) with ethyl acetate (3 x 700 ml) in a separating funnel (5 L). The combined extracts were dried over anhydrous

sodium sulphate. After removal of the ethyl acetate *in vacuo* gummy mixtures were obtained. The yields of crude products are summarised in **Table 21**.

A116 gave yellowish crystals (0.58 g), which were triturated with acetone and recrystallised from methanol to give white hairy needles (12 mg) of cytochalasin D. TLC studies of the individual crude extract indicated that the four endophytes (**B127R**, **B338**, **B231b** and **B115**) produced the same components.

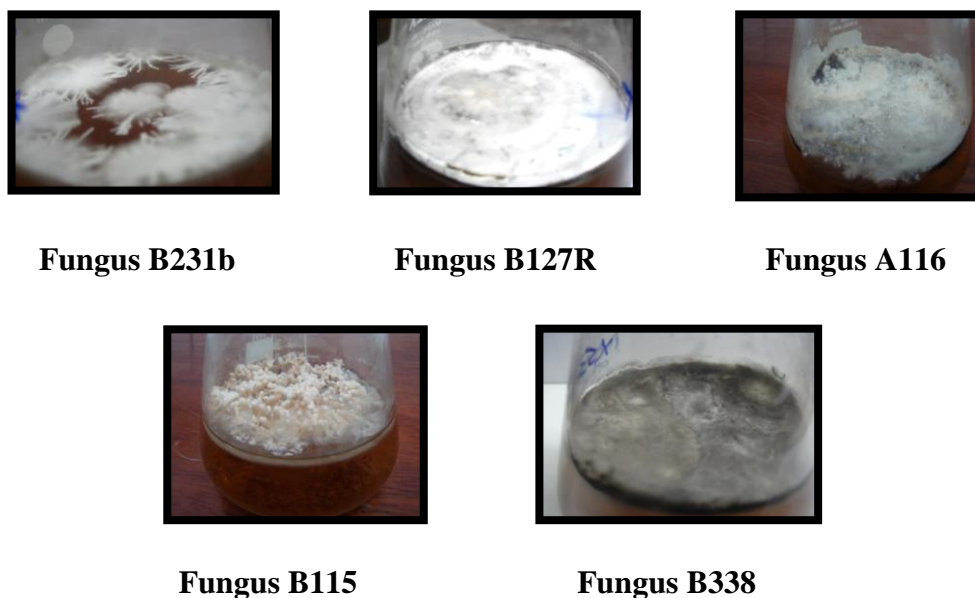


Figure 83 Endophytic fungi growing in conical flasks after 10 days

Table 21 Summary of endophytes crude extracts

Fungus	Crude Yield	Description
B127R	10.1 g	Brown gum
B231b	6.2 g	Brown gum
B338	4.6 g	Gummy brown liquid
B115	2.7 g	Brown gum
A116	0.58 g	Yellowish crystals

7.7.1 Isolation of Cytochalasin D

The crude extract of **B127R** (10.1 g) was taken up into ethyl acetate (20 ml) producing a thick creamy solution. The solution was left to set aside for 24 h. A fine white solid (106 mg) precipitated from solution. The solid was filtered off and recrystallised from methanol to give cytochalasin D as colourless needles (79 mg), mp 230-232 °C, ES $[M+H]^+$ m/z 508 (consistent with $C_{30}H_{37}NO_6$), $[\alpha]_D^{25} -12.5^\circ$ (c 1, dioxane), R_f 0.24 in solvent system; toluene, ethyl acetate and acetic acid (50:49:1), $IR_{ATR} \nu_{max} cm^{-1}$ 3419, 1741 and 1692, [lit²⁵, mp 267-270 °C], δ_H (C_5D_5N) 0.76 (3H, d, J 6.7 Hz, 11-CH₃), 0.97 (3H, d, J 6.5 Hz, 22-CH₃), 1.49 (3H, s, 23-CH₃), 1.90-1.93 (1H, m, 15-Ha), 2.41 (3H, s, 25-CH₃), 2.42 (1H, s, 4-H), 2.58-2.63 (1H, m, 15-Hb), 2.66-2.70 (1H, m, 16-H), 2.80-2.83 (2H, m, 10-H), 3.00 (1H, s, 5-H), 3.35-3.39 (1H, m, 8-H), 3.54 (1H, s, 3-H), 4.40 (1H, d, J 10.3 Hz, 7-H), 4.98 (1H, s, 7-OH), 5.04 (1H, s, 12-Ha), 5.40 (1H, s, 12-Hb), 5.54-5.59 (1H, m, 19-H), 5.60-5.61 (1H, m, 13-H), 6.18 (1H, s, 21-H), 6.30-6.35 (1H, m, 14-H), 6.83 (1H, dd, J 2.5, 13.3 Hz, 20-H), 7.20-7.35 (5H, m, aromatic protons), 9.08 (1H, s, N-H), δ_C (C_5D_5N) 13.59 (CH₃, C-11), 19.34 (CH₃, C-22), 20.51 (CH₃, C-23), 24.60 (CH₃, C-25), 33.13 (CH, C-5), 38.54 (CH₂, C-15), 42.43 (CH, C-16), 45.49 (CH₂, C-10), 47.86 (CH, C-8), 50.07 (CH, C-4), 53.98 (C, C-9), 54.35 (CH, C-3), 71.26 (CH, C-7), 77.98 (CH, C-21), 78.38 (C, C-18), 112.16 (=CH₂, C-12), 126.79 (HC=C, C-4'), 127.82 (HC=C, C-13), 128.79 (HC=C x 2, C-3' & C-5'), 129.89 (HC=C x 2, C-2' & C-6'), 132.18 (HC=C, C-19), 133.81 (HC=C, C-20), 132.75 (HC=C, C-14), 138.43 (C, C-1'), 151.61 (C, C-6), 170.45 (C, C-24), 175.09 (C, C-1), 210.86 (C, C-17).

7.7.2 Isolation of Coriloxin

After the isolation of cytochalasin D, the remaining filtrate was concentrated *in vacuo* to yield a brownish gummy liquid (5.0 g), which was triturated with acetone

and set aside overnight. This afforded dark brown crystals which were recrystallised twice from toluene to yield optically active colourless needles of coriloxin (103 mg), mp 153-155 °C, ES $[M+H]^+$ m/z 171 (consistent with $C_8H_{10}O_4$), $[\alpha]$ -98.4° (c 1, in EtOH), [lit³⁶, mp 153-155 °C, $[\alpha]$ -100° (c 0.33, in EtOH)], R_f 0.13 in solvent system; toluene, ethyl acetate and acetic acid (50:49:1), IR_{ATR} $\nu_{max}cm^{-1}$ 3403, 1648 and $1607\ cm^{-1}$, δ_H ($CDCl_3$) 1.62 (3H, s, 7-CH₃), 2.68 (1H, d, J 6.2 Hz, 4-OH), 3.30 (1H, d, J 1.7 Hz, 6-H), 3.73 (3H, s, 8-CH₃), 4.46 (1H, d, J 6.2 Hz, 4-H), 5.23 (1H, d, J 1.7 Hz, 2-H), δ_c ($CDCl_3$) 19.02 (CH₃, C-7), 56.69 (CH₃, C-8), 59.55 (C, C-5), 60.65 (CH, C-6), 69.21 (CH, C-4), 98.29 (CH, C-2), 171.54 (C, C-3), 193.56 (C=O, C-1). The compound showed no phenolic properties with diazotised *p*-nitroaniline spray reagent; however it gave a yellowish green colouration after spraying with anisaldehyde spray reagent and heating 2-3 min at 110 °C.

7.7.3 Isolation of *S*-Mellein

After removal of coriloxin, the crude product had a dry weight of (3.3 g). Two portions (1 g each) of this brownish viscous liquid were chromatographed on two large thick layer plates (20 x 80 cm); (1.0 g) on each plate. The plates were developed in a tank containing toluene, ethyl acetate and acetic acid (500:490:10). As the plates developed, they were removed and air-dried and then strips (1.0 cm) at the edges were sprayed with diazotised *p*-nitroaniline spray reagent to detect the bands. (Band 1), (R_f 0.91) in solvent system toluene, ethyl acetate and acetic acid (50:49:1) was scraped off and washed with ethyl acetate which was removed *in vacuo* to yield a yellow oil, which was triturated with *n*-hexane to give a powdery solid (7.5 mg). This was recrystallised from *n*-hexane to yield *S*-mellein as colourless plates (3 mg), mp 52-53 °C, ES $[M+H]^+$ m/z 179 (consistent with $C_{10}H_{10}O_3$), $[\alpha]$ $+87^\circ$ (c 1, in $CHCl_3$), [lit⁹¹, mp 56-58 °C, $[\alpha]$ $+88^\circ$ (c 0.27, in $CHCl_3$)], IR_{ATR} ν_{max}

cm⁻¹ 3187, 1662, δ_{H} (CDCl₃) 1.51 (3H, d, J 6.3 Hz, 11-CH₃), 2.91 (2H, d, J 7.0 Hz, 4-CH₂), 4.70-4.73 (1H, m, 3-H), 6.67 (1H, d, J 7.3 Hz, 7-H), 6.87 (1H, d, J 8.4 Hz, 5-H), 7.39 (1H, t, J 7.5 Hz, 6-H), 11.02 (1H, s, OH), δ_{C} (CDCl₃) 20.88 (CH₃, C-11), 34.73 (CH₂, C-4), 76.21 (O-CH, C-3), 116.36 (C=CH, C-7), 118.00 (C=CH, C-5), 136.25 (C=CH, C-6), 108.41 (C, C-9), 139.50 (C, C-10), 162.31 (C, C-8), 170.07 (C, C-1).

7.7.4 Isolation of *cis*-4-Hydroxymellein

The next UV active band on the preparative TLC plate at R_{f} 0.71 developed in solvent system; toluene, ethyl acetate and acetic acid (50:49:1) was removed and washed with ethyl acetate to yield a yellow solution. The solvent was removed *in vacuo* to afford yellowish needles, which were recrystallised from toluene to yield *cis*-4-hydroxymellein as fine colourless prisms (87 mg), mp 112-117 °C, ES $[\text{M}+\text{H}]^+$ m/z 195 (consistent with C₁₀H₁₀O₄), $[\alpha]_{\text{D}}^{25} +48^\circ$ (c 1, in MeOH), $[\text{lit}^{96}]$, mp 118-119 °C, $[\alpha]_{\text{D}}^{25} +37.4^\circ$ (c 0.33, in MeOH), IR_{ATR} ν_{max} cm⁻¹ 3430, 3178, 2997, 1642 and 1615, δ_{H} (CDCl₃) 1.57 (3H, d, J 6.6 Hz, 11-CH₃), 2.04 (1H, d, J 6.4 Hz, 4-OH), 4.68 (1H, dd, J 6.1, 1.8 Hz, 4-H), 4.69 (1H, qd, J 6.6, 2.0 Hz, 3-H), 6.90 (1H, d, J 7.3 Hz, 5-H), 7.01 (1H, dd, J 8.4, 0.9 Hz, 7-H), 7.50 (1H, t, J 7.3 Hz, 6-H), 10.98 (1H, s, OH), δ_{C} (CDCl₃) 16.15 (CH₃, C-11), 67.39 (CH, C-4), 78.27 (CH, C-3), 106.94 (C, C-9), 118.39 (C=CH, C-7), 118.71 (C=CH, C-5), 136.92 (C=CH, C-6), 140.58 (C, C-10), 162.25 (C, C-8), 169.26 (C, C-1).

7.8 Isolation of Cytochalasin D from fungus B127R mycelium

The air-dried mycelium was pulverised (53.9 g) and soxhlet extracted with chloroform (400 ml) for 16 h to give a brownish gum (7.6 g). The gum was dissolved in warm ethyl acetate (15 ml) and set aside for 24 h. A white solid (2.3 g) was obtained after the solution was filtered off. TLC studies showed an opaque spot

with diazotised *p*-nitroaniline spray reagent, which indicated a cytochalasin. The ^1H and ^{13}C NMR spectra ($\text{C}_5\text{D}_5\text{N}$) indicated that the compound was cytochalasin D, which was isolated and characterised from the culture medium of fungus **B127R**.

7.9 Secondary metabolites from the fungus X.B315 on malt extract medium

The fungus **X.B315** was static sub-cultured on aqueous malt extract medium into five conical flasks (250 ml) for 14 days. It produced a white mycelium with a black colouration underside; no fruiting bodies were observed **Figure 84**.



A-in a conical flask after 2 weeks

B-in Thompson bottle after 4 weeks

Figure 84 Fungus X.B315 on malt extract-glucose mixture

The fungus was then transferred into Thompson bottles (15 x 1L) each bottle containing about (500 ml) of the medium solution. The mature fungus produced a white gelatine-like mycelium with black underside.

The matured cultures were harvested and the mycelia were recovered from the brown culture medium by filtration through a muslin cloth and left to air-dry. The brownish filtrate was extracted with ethyl acetate (700 ml) three times in a separating funnel. The combined extracts were dried over anhydrous sodium sulphate. Evaporation of the solvent *in vacuo* yielded a malt crude extract (5.2 g).

The latter was screened using TLC for its content. The solvent system; toluene, ethyl acetate and acetic acid (50:49:1) used as eluent. The resultant plates showed that the crude extract was a mixture of three components; two spots (R_f 0.92 & 0.64)

developed an intense yellow colouration with diazotised *p*-nitroaniline spray reagent, whilst the third spot at R_f 0.32 developed a yellowish brown colouration. The spot at R_f 0.32 showed an intense yellow colouration with acetic acid, anisaldehyde and sulphuric acid (98:1:1) spray reagent, which turned to red after heating 2-3 min at 110 °C.

7.9.1 Isolation of Cytochalasin D from fungus X.B315

The crude extract (5.2 g) was triturated with ethyl acetate and left overnight. A white precipitate was obtained. The white solid was filtered off and recrystallised from methanol to yield cytochalasin D as colourless needles (65 mg), mp 238-240 °C, ES $[M+H]^+$ m/z 508. This compound showed an opaque spot when treated with diazotised *p*-nitroaniline spray reagent at R_f 0.27 on TLC. The 1H NMR spectrum (C_5D_5N) showed a characteristic N-H signal at δ_H 9.01. The spectral and physical data of this compound confirmed this compound as cytochalasin D the prior identification of which is described in **Chapter 2**.

7.9.2 Isolation of *R*-Mellein from fungus X.B315

After removal of cytochalasin D, the ethyl acetate crude extract (3.7 g) was chromatographed on four PTLC plates (20 cm x 80 cm); (1 g) on each plate. Each plate was prepared by mixing Merck Kieselgel GPF254 silica gel (100 g) with distilled water (270 ml) in a beaker (1 L) and left for 45 min to settle before spreading on the clean glass PTLC plate. After applying the extract on the plate, it was developed in a large tank containing toluene, ethyl acetate and acetic acid (500:450:10). After the plates developed, they were removed and air-dried and a band at each end of each plate (1.0 cm) was sprayed with diazotised *p*-nitroaniline spray reagent to detect the bands. The UV active band at R_f 0.92 was recovered and washed with ethyl acetate to produce a yellow oil (35 mg). Trituration of the oil with

n-hexane gave a white solid (12 mg), which was recrystallised twice from the same solvent to yield *R*-mellein as colourless plates (3 mg), mp 52-53 °C, ES $[M+H]^+$ m/z 179, $[\alpha]_D^{25}$ -100° (*c* 1, in EtOH), [lit.³⁹ mp 56-58 °C, $[\alpha]_D^{25}$ -80° (*c* 0.02, in EtOH)], δ_H (CDCl₃) 1.52 (3H, d, *J* 6.3 Hz, 11-CH₃), 2.93 (2H, d, *J* 7.0 Hz, 4-CH₂), 4.69-4.75 (1H, m, 3-H), 6.67 (1H, d, *J* 7.3 Hz, 7-H), 6.87 (1H, d, *J* 8.4 Hz, 5-H), 7.39 (1H, t, *J* 7.5 Hz, 6-H), 11.02 (1H, s, OH), δ_C (CDCl₃) 20.88 (CH₃, C-11), 34.73 (CH₂, C-4), 76.21 (O-CH, C-3), 116.36 (C=CH, C-7), 118.00 (C=CH, C-5), 136.25 (C=CH, C-6), 108.41 (C, C-9), 139.50 (C, C-10), 162.31 (C, C-8), 170.07 (C, C-1).

7.9.3 Isolation of *cis,trans*-4-Hydroxymellein mixture from the fungus X.B315

The next UV active band on the PTLC plate, which had *R_f* 0.64, developed a strong orange colouration with *p*-nitroaniline spray reagent. It was isolated as a pale yellow oil and recrystallised from petroleum ether (bp 80-100 °C) as fine colourless needles (4 mg) and found to be a mixture of two 4-hydroxymellein stereoisomers (C₁₀H₁₀O₄), mp 121-125 °C, [lit.⁴⁰, 131-132 °C], ES $[M+H]^+$ m/z 195, δ_H (CDCl₃) 1.49 (3H, d, *J* 6.2 Hz, 11-CH₃), 1.56 (3H, d, *J* 6.7 Hz, 11-CH₃), 2.14 (1H, s, OH), 4.57 (1H, m, 3-H), 4.60 (1H, m, 3-H), 4.55 (1H, m, 4-H), 4.56 (1H, m, 4-H), 6.90 (1H, m, 5-H), 6.91 (1H, m, 5-H), 7.00 (1H, m, 6-H), 7.01 (1H, m, 6-H), 7.49 (1H, m, 7-H), 7.52 (1H, m, 7-H), 10.95 (1H, s, OH), 10.98 (1H, s, OH), δ_C (CDCl₃) 16.09, 18.00 (CH₃, C-11), 67.29, 69.19 (CH, C-4), 78.29, 80.05 (CH, C-3), 106.73, 106.90 (C, C-9), 116.35, 117.87 (C=CH, C-7), 118.39, 118.60 (C=CH, C-5), 136.89, 136.97 (C=CH, C-6), 140.58, 141.27 (C, C-10), 162.04, 162.14 (C, C-8), 168.59 169.29 (C, C-1).

7.9.4 Isolation of Phloroglucinol from fungus X.B315

A compound, which gave a violet spot under visible light on the PTLC plate at *R_f* 0.32, developed a yellowish brown colouration with *p*-nitroaniline spray reagent, whilst it developed an intense yellow colouration with anisaldehyde spray reagent,

which turned to red after heating 2-3 min at 110 °C. The band was recovered and washed with ethyl acetate to produce a brown powder (420 mg) after evaporation of the solvent *in vacuo*. The powder was recrystallised twice from H₂O to yield phloroglucinol as colourless crystals (350 mg), mp 214-216 °C, ES [M+H]⁺ *m/z* 127 (consistent with C₆H₆O₃), IR_{ATR} ν_{\max} cm⁻¹ 3461, 3160 and 1609, δ_{H} (acetone-d₆) 5.84 (H x 3, s), 8.08 (OH x 3, s), δ_{C} (acetone-d₆) 94.55 (CH x 3), 159.33 (=C-OH x 3).

7.10 Isolation of Phloroglucino from fungus **X.B315** mycelium

The air-dried mycelium (65.5 g) was pulverised. The sample was packed into a soxhlet thimble, which was placed into a soxhlet apparatus. It was extracted with chloroform (400 ml) for 16 h. After evaporation of the solvent *in vacuo*, a brown crude extract (5.6 g) was obtained. The extract was then analysed for its content by TLC. A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. A visible violet spot was detected on a TLC plate at R_f 0.32. The spot developed a yellowish brown colouration with diazotised *p*-nitroaniline spray reagent, whilst it developed a yellow colouration with anisaldehyde spray reagent, which turned to red after heating 2-3 min at 110 °C. The crude extract was warmed with H₂O (15 ml) for 10 min. The solution was filtered off and the filtrate was left to cool. Filtration gave colourless crystals of phloroglucinol (450 mg). It appears that phloroglucinol is a major metabolite produced by fungus **X.B315**.

7.11 Secondary metabolites from fungus **X.B315** on potato extract

Analogous procedures to those described above for the isolation of secondary metabolites of **X.B315** grown on malt extract-glucose mixture were applied to the ethyl acetate crude extract of the same fungus grown on potato-yeast-extract-glucose mixture. It was found that the fungus **X.B315** grown on potato-yeast-extract-glucose mixture produced the same metabolites, but in higher yield. The mycelium of this

fungus also produced phloroglucinol. The potato extract was prepared by boiling (1 kg) white potatoes in distilled water (5 L) for 30 min. The solution was filtered off through a filter paper.

7.12 Secondary metabolites from the endophytic fungus X.62

The fungus **X.62** was isolated from palm tree leaves in Thailand and classified as a *Xylaria* endophyte. It was received in Petri dishes as shown in **Figure 85**. The front view of the Petri dish showed a white mycelium. The reverse side of the fungus was also white; but with a black ring round the edge of the dish.



Figure 85 Fungus X.62 as received from Thailand

The fungus was static sub-cultured into four conical flasks (250 ml) for 2 weeks. It produced a white mycelium **Figure 86**.



Figure 86 Fungus X.62 in conical flask after 2 weeks

The fungus was then cultured on a malt-yeast extract-glucose mixture into Thompson bottles (15 x 1L) for 8 weeks. During the first 4 weeks, it produced a white

mycelium with black areas underside. The matured cultures were harvested and the mycelia were recovered by filtration through a muslin cloth and left to air-dry. The aqueous filtrate was extracted with ethyl acetate in a separating funnel (5 L). The combined fractions were dried over anhydrous sodium sulphate. After removal of the solvent *in vacuo*, a brown crude extract was obtained (1.2 g).

TLC analysis of the crude extract was conducted in different solvent systems. The resultant plates showed that the solvent systems; toluene, ethyl acetate and acetic acid (50:49:1), chloroform and methanol (95:5) and petroleum ether (bp 60-80 °C), ethyl acetate and acetic acid (50:49:1) showed that the crude extract contained a complex mixture of different compounds with different R_f values. When the plates were sprayed with *p*-nitroaniline spray reagent opaque spots were revealed. ^1H NMR spectroscopy ($\text{C}_5\text{D}_5\text{N}$) showed that the extract contained a mixture of cytochalasins according to four N-H signals. Several attempts to isolate these compounds by chromatographic techniques failed.

7.12.1 Isolation of 19,20-Epoxychochalsin C from X.62 mycelium

The air-dried mycelium (79.3 g) was pulverised. The powdered mycelium was packed firmly into a soxhlet thimble, which was placed into a soxhlet apparatus. The sample was extracted with chloroform (400 ml) for 16 hr. After the evaporation of the solvent *in vacuo*, a brown gummy solid was obtained (5.0 g). The extract was analysed for its content by TLC.

A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. A single spot under short wave UV (245 nm) with R_f 0.42 was detected on a TLC plate. When the plate was sprayed with *p*-nitroaniline reagent an opaque spot was developed at R_f 0.42. The ethyl acetate extract was triturated with ethyl acetate and left overnight. A white solid was obtained. The solid was filtered off and

recrystallised from methanol to yield 19,20-epoxycytochalasin C as white needles (373 mg), mp 266-270 °C, ES $[M+H]^+$ m/z 524 (consistent with $C_{30}H_{37}NO_7$), $[\alpha]_D^{25}$ -6.4° (c 1, in $CHCl_3$), $[\alpha]_D^{29}$ -6.8° (c 0.25, in $CHCl_3$), IR_{ATR} ν_{max} cm^{-1} 3423, 1744 and 1697, δ_H (C_5D_5N) 0.98 (3H, d, J 6.5 Hz, 22-CH₃), 1.94 (1H, dd, J 11.1, 12.2 Hz, 15-Ha), 1.24 (3H, s, 12-CH₃), 1.59 (3H, s, 23-CH₃), 1.83 (3H, s, 11-CH₃), 2.10 (3H, s, 25-CH₃), 2.16 (1H, s, 8-H), 2.60-2.63 (1H, m, 15-Hb), 2.82-2.84 (2H, m, 10-H), 2.85-2.88 (1H, m, 16-H), 3.17 (1H, d, J 7.3 Hz, 4-H), 3.59 (1H, t, J 7.2 Hz, 3-H), 3.75 (1H, s, 19-H), 4.20 (1H, s, 20-H), 4.40 (1H, s, 7-H), 4.97 (1H, s, OH), 5.85-5.89 (1H, m, 13-H), 6.26 (1H, s, 21-H), 6.73-6.77 (1H, m, 14-H), 7.20-7.35 (5H, m, aromatic protons), 9.46 (1H, s, N-H), δ_C (C_5D_5N) 14.78 (CH₃, C-11), 17.06 (CH₃, C-12), 19.05 (CH₃, C-22), 20.38 (CH₃, C-23), 22.55 (CH₃, C-25), 38.35 (CH₂, C-15), 42.00 (CH, C-16), 45.28 (CH₂, C-10), 50.08 (CH, C-3), 51.03 (CH, C-4), 52.82 (CH, C-8), 54.11 (O-CH, C-19), 60.75 (C, C-9), 61.45 (O-CH, C-20), 68.89 (CH, C-7), 73.36 (CH, C-21), 77.16 (C, C-18), 126.58 (C, C-5), 126.81 (HC=C, C-4'), 128.84 (HC=C x 2, C-3' & C-5'), 129.88 (HC=C x 2, C-2' & C-6'), 132.21 (HC=C, C-13), 132.89 (HC=C, C-14), 133.72 (C, C-6), 138.74 (C, C-1'), 170.84 (C, C-24), 175.35 (C, C-1), 216.19 (C, C-17).

7.13 Secondary metabolites from fungus *Engleromyces sinensis*

The samples of this fungus, which belongs to the family Hypocreaceae, were collected on Snow Mountain at ca 2700 m, Lijiang, Yunnan province in China. The fungus had yellowish flesh with a hard yellowish-brown exterior crust **Figure 87**. The old herbarium material was hard and horny, but became soft when soaked in water. The hard crust of the fungus was cut into pieces and pulverised using a grinder. The powdered sample (60.5 g) was packed firmly into a soxhlet thimble, which was placed into a soxhlet apparatus. The sample was extracted with four

different solvents (400 ml of each) for 24 h. The hexane fraction yielded yellowish oil (1.4 g), whilst the chloroformic fraction yielded a light brown gum (0.5 g). The ethyl acetate fraction yielded a light brown gum (43 mg) and the methanol fraction yielded a dark brown gum (7.1 g).

The different extracts were then examined for their metabolite profile by TLC. A solvent system of toluene, ethyl acetate and acetic acid (50:49:1) was used as eluent. Hexane extract showed a UV active spot with R_f 0.9, whilst chloroform extract did not show any UV active spots. Ethyl acetate and methanol extracts showed matching patterns of a single spot under UV Lamp at R_f 0.32. When the plates were dried, the both extracts were sprayed with *p*-nitroaniline reagent, the spot developed an opaque colouration, suggesting that the ethyl acetate and methanol extracts contained the same component.



Figure 87 Fungus *Engleromyces sinensis* as received from China

7.13.1 Isolation of Engleromycin acetate

A. Ethyl acetate extract

A white precipitate formed in the ethyl acetate extract. The solid (23 mg) was filtered off and recrystallised twice from ethyl acetate to yield engleromycin acetate as colourless needles (10 mg), mp 265-269 °C [lit.,¹¹⁹ mp 226-228 °C], ES $[M+H]^+$ m/z 524, (consistent with $C_{30}H_{37}NO_7$), $[\alpha]_D^{25}$ -220° (*c* 1, in $CHCl_3$), [lit.,³⁰ $[\alpha]_D^{25}$

-228° (c 0.035, in CHCl₃), IR_{ATR} ν_{\max} cm⁻¹ 3748, 3378, 1754 and 1670, δ_{H} (C₅D₅N) 0.58 (3H, d, J 6.6 Hz, 11-CH₃), 0.91 (3H, d, J 6.5 Hz, 22-CH₃), 1.51 (3H, s, 23-CH₃), 1.91 (1H, d, J 6.5 Hz, 15-Ha), 2.07 (3H, s, 25-CH₃), 2.45 (1H, s, 4-H), 2.71-2.76 (1H, m, 15-Hb), 2.77-2.79 (1H, m, 16-H), 2.80-2.89 (2H, m, 10-H), 3.00-3.10 (1H, m, 5-H), 3.20 (1H, s, 8-H), 3.46 (1H, s, 3-H), 3.65 (1H, d, J 2.5 Hz, 19-H), 4.23 (1H, d, J 2.5 Hz, 20-H), 4.32 (1H, d, J 10.3 Hz, 7-H), 4.86 (1H, s, 7-OH), 5.27 (1H, s, 12-Ha), 5.59 (1H, s, 12-Hb), 5.91-5.96 (1H, m, 13-H), 6.16 (1H, s, 21-H), 6.49-6.56 (1H, m, 14-H), 7.20-7.35 (5H, m, aromatic protons), 9.13 (1H, s, N-H), δ_{C} (C₅D₅N) 13.36 (CH₃, C-11), 18.95 (CH₃, C-22), 20.36 (CH₃, C-23), 22.71 (CH₃, C-25), 32.93 (CH, C-5), 38.19 (CH₂, C-15), 42.46 (CH, C-16), 45.43 (CH₂, C-10), 47.33 (CH, C-8), 50.57 (CH, C-4), 53.66 (C, C-9), 53.96 (CH, C-3), 54.37 (CH, C-19), 60.70 (CH, C-20), 71.37 (CH, C-7), 75.11 (CH, C-21), 77.30 (C, C-18), 112.03 (=CH₂, C-12), 126.78 (HC=C, C-4'), 128.77 (HC=C x 2, C-3' & C-5'), 129.97 (HC=C x 2, C-2' & C-6'), 132.04 (HC=C, C-13), 132.53 (HC=C, C-14), 138.38 (C, C-1'), 151.39 (C, C-6), 170.62 (C, C-24), 175.04 (C, C-1), 216.37 (C, C-17).

B. Methanol extract

The crude extract (7.1 g) was triturated with methanol-water (50:50) and left overnight. A brown solid was obtained. The brown solid (50 mg) was filtered off and recrystallised three times from the same solvent system to yield engleromycin acetate as colourless needles (7 mg).

7.14 Secondary metabolites from fungus *X. polymorpha*

The fungus *X. polymorpha*, which is a plant pathogen, was collected from Thailand. The fungus was static sub-cultured for two weeks in 5 conical flasks (250 ml) containing an aqueous malt extract medium. It produced a white mycelium with a

light brown gelatinous underside. It started fruiting after 10 days and developed light brown cylindrical shaped-stromata with unbranched tips **Figure 88**.



Figure 88 Fungus *X. polymorpha* in a conical flask after 10 days

The fungus was then sub-cultured into Thompson bottles (15 x 1L), each bottle containing (500 ml) of a malt-yeast extract-glucose mixture for 8 weeks. The matured cultures were harvested and the mycelia were recovered by filtration through a muslin cloth. The brownish filtrate was extracted with ethyl acetate in a separating funnel (5 L). The combined extracts were dried over anhydrous sodium sulphate. After the evaporation of the solvent *in vacuo*, a brown gummy mixture was obtained (2.3 g). The ethyl acetate crude extract (2.3 g) was chromatographed over Merck Kieselgel 60, column (3 cm x 80 cm). The column was eluted with petroleum ether (bp 60-80 °C), ethyl acetate and acetic acid (30:70:1) and fractions of 3.0 ml were collected using an automated fraction collector.

7.14.1 Isolation of (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'methylphenyl)-2-methoxybut-3-enoic acid

The component recovered (tubes 20-55) was a yellow oil, which was triturated with ethyl acetate to yield a white solid (13 mg), which was recrystallised from the same solvent to yield (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'methylphenyl)-2-methoxybut-3-enoic acid as colourless crystals (7 mg), mp 160-163 °C, ES $[M+H]^+$ m/z 281 (consistent with $C_{14}H_{16}O_6$), $[lit^{34}]$, mp 160-165°C], R_f 0.4 in solvent system;

petroleum ether (60-80 °C), ethyl acetate and acetic acid (30:70:1), IR_{ATR} ν_{\max} cm⁻¹ 3300, 1774 and 1628, δ_{H} (C₅D₅N) 2.28 (3H, s, 9'-CH₃), 2.43 (3H, s, 8'-CH₃), 3.48 (3H, s, 5-CH₃), 4.79 (1H, dd, *J* 6.6, 1.1 Hz, 2-H), 7.49 (1H, s, 4'-H), 7.60 (1H, dd, *J* 16.2, 6.6 Hz, 3-H), 7.75 (1H, dd, *J* 16.2, 1.1 Hz, 4-H), 14.06 (1H, s, 2'-OH), δ_{C} (C₅D₅N) 17.08 (CH₃, C-9'), 26.08 (CH₃, C-8'), 56.95 (CH₃, C-5), 83.58 (CH, C-2), 112.35 (C, C-1'), 113.02 (C, C-3'), 116.80 (C, C-5'), 124.07 (CH, C-4), 129.91 (CH, C-3), 132.35 (CH, C-4'), 162.27 (C, C-2'), 162.70 (C, C-6'), 173.63 (C, C-1), 203.37 (C, C-7').

Chapter 8 Summary

The *Xylariaceae* is a well-known family for the wide biological diversity of many of its species and genera. It has also been found to be an impressive source of metabolites of which many have proved to be novel structures²⁷. The genus *Xylaria* with worldwide distribution in tropical and sub-tropical areas has proved to be one of the most rewarding with 30 new compounds isolated from 30 species²⁷.

Endophytic *Xylaria* are an important source of novel compounds¹²⁵ and bioactive agents, which can lead to the discovery of new drugs for the pharmaceutical and agricultural industries⁴⁷.

In this project the samples were collected from *Xylariaceae* as well as endophytic fungi and the fungi were static sub-cultured on different culture media.

In this study, secondary metabolites from five endophytic *Xylaria* species were collected from the Trad Province in Southern Thailand. The structures of the compounds have been elucidated by chemical and spectroscopic methods.

The endophytic fungi **B127R**, **B338**, **B115**, **B231b** produced cytochalasin D, coriloxin, *S*-mellein and (3*R*,4*R*)-4-hydroxymellein **Figure 89**, suggesting that the four fungi were the same species, whilst fungus **A116** produced cytochalasin D.

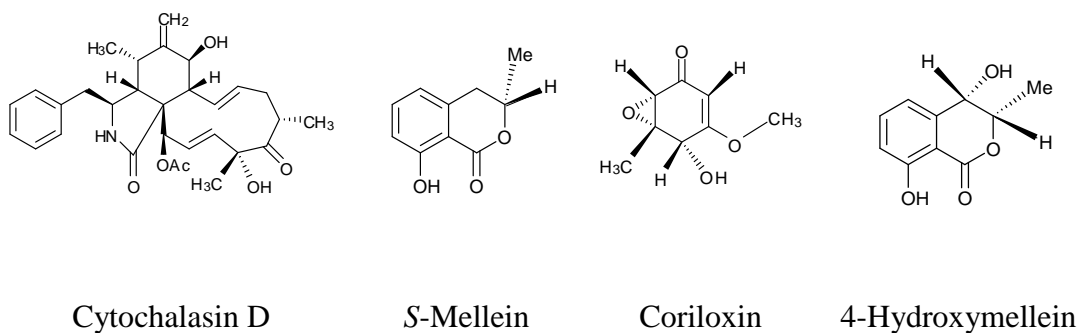


Figure 89 Secondary metabolites isolated from endophytic fungi

The fungus **X.B315** was cultured on two different media; malt extract and potato-yeast extract mixture and found to produce; cytochalasin D, *R*-mellein, a mixture of two isomers of the 4-hydroxymellein and phloroglucinol **Figure 90**.

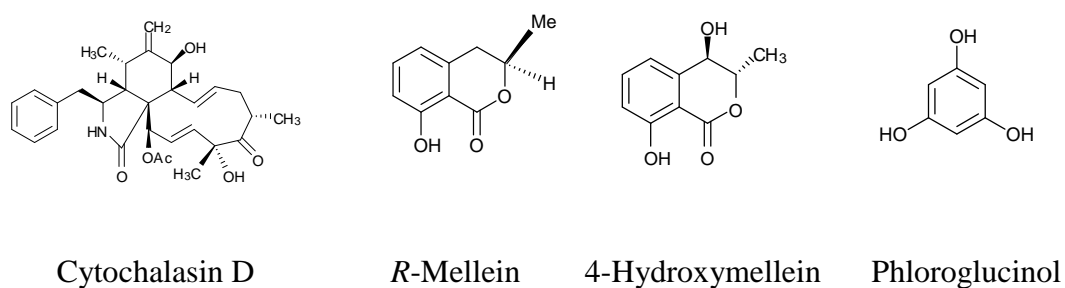
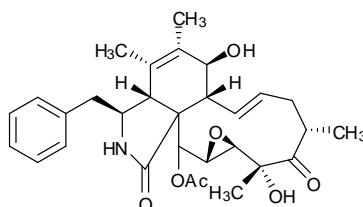


Figure 90 Secondary metabolites isolated from fungus X.B315

The fungus **X.62**, a *Xylaria* endophyte, produced 19,20-epoxycytochalasin C **Figure 91**.

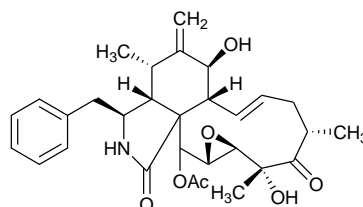


19,20-Epoxycytochalasin C

Figure 91 Secondary metabolite from fungus X.62

Fungus *Engleromyces sinensis*, the second species in the genus after *Engleromyces goetzei*, produced 19,20-epoxycytochalasin D, also known as engleromycin acetate

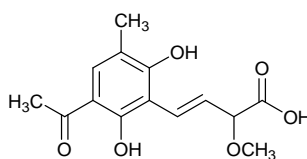
Figure 92.



Engleromycin acetate

Figure 92 Secondary metabolite from fungus *Engleromyces sinensis*

The fungus *X. polymorpha*, a plant pathogen, most likely produced globoscin³⁴, which slowly rearranged in pyridine and to give (3E)-4-(3'-acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid **Figure 93**.



(3E)-4-(3'-Acetyl-2',6'-dihydroxy-5'-methylphenyl)-2-methoxybut-3-enoic acid

Figure 93 Pyridine rearrangement product

During the course of the work of fungus *X. polymorpha*, a second metabolite of unknown structure was isolated in insufficient quantities to allow full characterisation. One future area of work the fungus *X. polymorpha* would be recultured on a large scale to isolate and identify this second component. Initial spectroscopic data suggested it could be a novel structure.

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